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CONTENTS

No. 1, July, 1939

No. 2, August, 1939

No. 3, September, 1939

No. 4, October, 1939

No. 5, November, 1939

No. 6, December, 1939

William Hallock Park[1]

1863–1939

When a man like William H. Park dies, something is irretrievably lost which far transcends the contributions which he might still have made to his science. There is built up, in the course of the lives of men of his kind, an accumulated judgment, a penetration and a view of professional standards which together constitute a personality—unique and entirely individual. Among the distinguished men of the generation which founded modern bacteriology in our country—a group which included preëminently Theobald Smith, Vaughan, Ricketts, Buxton, Dunham, Flexner and some others—Park stood out as the one who particularly carried his efforts toward the public health application of bacteriological methods. Trained at the College of Physicians and Surgeons in New York and looking forward to a career of medical practice, his interests were turned to bacteriology by a sojourn in Vienna, where the young science was growing into the early years of its Golden Age. Returning to New York, Park became associated with T. M. Prudden, one of the first in our country to develop bacteriology as a separate discipline in a medical school, and Hermann M. Biggs, to whose vision New York state was to owe its sound public health development. It is no small testimony to the wisdom of these men that they selected Park as the organizing director of the New York City Health Department Laboratory. He accepted this position in 1893 and retained it until 1936, when he became Director Emeritus. During these forty-three years of continuous service, Park developed a laboratory which stands as a landmark in the history of public health organization and which served for a long time as a model for other city and state institutions. His genius

[1] President, Society of American Bacteriologists, 1912.

lay particularly in his capacity for combining sound research with practical application and in maintaining the public health purposes of his work without any compromise in the rigidity of scientific standards. By these rare qualities he became a significant figure in American public health development, occupying—in the writer's opinion—a position analogous to that which his distinguished contemporary, Dr. Welch, held in regard to the training of academic investigators.

While his vigilance for the useful application of bacteriological discovery led him into wide ranges of inquiry, including milk and infantile diarrhea, poliomyelitis, meningitis, scarlet fever and tuberculosis, Park's permanent place in the history of bacteriology will rest on his work in diphtheria. In this it is not an exaggeration to say that, although his contributions were in the direction of prevention more than in that of fundamental biology, he ranks with Klebs, Loeffler, Behring and Roux. His early studies on diagnosis, on the carrier state and—with Biggs—on antitoxin treatment, while not original in principle, carried knowledge and methods into medical practice in a manner not elsewhere achieved at that time. Later, his contributions to antitoxin production and purification, and his work on active immunization with toxin-antitoxin mixtures mark new epochs in the conquest of the disease. Most extraordinary in these accomplishments is the fact that, while directing and inspiring studies of this quality, he was able to organize mass application on a scale never before attempted.

The children of America—and of the world, for that matter—owe Dr. Park the greatest debt. He gave them clean milk, and he protected them from diphtheria. One need not be sentimental, remembering Dr. Park's gentleness and shy temperament, to take particular pleasure in this thought. It may well be that this choice of problems was not accidental.

His heart was in his work on equal terms with his mind. His modest words of 1933: "I have done nothing alone" may be taken literally, as they can be for any man who has directed a large organization. But this need not obscure the fact that he was the one who attracted the unusually able and singleminded men and women who worked with him; that his was the inspira-

tion; and that his spirit of thoroughness, integrity and good judgment pervaded the institution he directed.

Since this is to be read by bacteriologists, it is not necessary to speak of his books, his influence on scientific societies, nor of his tireless service on committees concerned with the perfection of public health organization. Nor need we make a point of his public and academic honors, as we might in the cases of lesser men.

Many loved Park, no one envied him and we, who know, take warm-hearted pride in the things we owe him. He was one of that small group who brought modern bacteriology to America; and he died, still in the first rank, still keenly interested and untired, one of those happy few who were destined never to know old age in the sense of intellectual retirement. All of his work was important, some of it of permanent value in the records of our science. And the example of his kindness, integrity and intelligent modesty will continue to set standards in many laboratories as long as any of those of us who were among his pupils may survive him.

HANS ZINSSER.

FACTORS GOVERNING THE DEVELOPMENT OF VARIATIONAL STRUCTURES WITHIN BACTERIAL COLONIES

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During the past twenty years the concept of bacterial dissociation or dissociative variation has grown at an increasingly rapid pace and, by inclusion of many of its pertinent features in current textbooks of bacteriology, it has at last gained admission to the society of legitimate subjects for investigation. While theories regarding the significance of variations have appeared from time to time, it has not yet become possible to formulate in precise terms an acceptable explanation of many of the every-day variation phenomena common to many species and families of bacteria and familiar to every student of the subject. The author wishes to call attention to this point by presenting an organized view of one small aspect of the problem, namely, certain variational structures known as "wedges," "sectors," or "outbursts" and secondary colonies. These structures are intimately associated with the mechanism of colonial variation.

The value of the colony as a tool in variation studies can hardly be overemphasized. The current accepted classification of M, S, R and G[2] phase cultures is in itself largely based on quite distinctive colony characteristics. Therefore many variants are most readily detected by observation of colonies. Moreover, the existence of macroscopically visible areas of culture differentiation in dissociating colonies is highly important be-

[1] Ralph R. Mellon, Director.

[2] Mucoid, smooth, rough and gonidial, the four primary colony types common to a great variety of species.

cause it makes possible the employment of a procedure of great value in variation studies, namely, selection.

Confining our attention to the case where variation takes place in the form of differentiated areas within the parent colony rather than appearing fully developed as isolated colonies of pure phase, we can classify the areas of variation into four types on the basis of their form and the manner of their development.

A. Wedges or sectors, which are more or less triangular areas with the apex directed toward the center of the colony and the body extending to the margin (fig. 1a).

B. Outbursts, which resemble wedges except that their outer border extends for some distance beyond the margin of the colony and spreads laterally after doing so (fig. 1b).

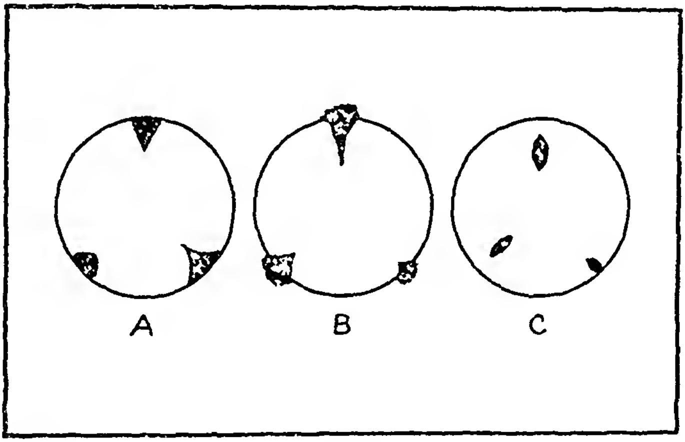

FIG. 1

C. Islands, which are isolated areas within a colony. They are usually elongated in the direction of the colonial radius, a form which suggests the nature of their origin as will be shown later (fig. 1c).

D. Secondary colonies, which are small colonies appearing on the surface or sometimes deep within the body of the parent colony usually late in its life. These colonies frequently represent a culture phase of lower order.[3]

[3] That is to say, a mucoid secondary on a smooth colony or a smooth or mucoid secondary on a rough colony.

They are in general quite small in proportion to the parent but sometimes eventually overgrow the original colony area.

In attempting to explain these forms on some unified basis, one may start with the basic assumption that all of these variant areas are derived from an initial variant cell whose distinguishing characteristics are transmitted to, and retained by, its progeny. In the case of all except the secondary colony, it may be further assumed that this variant cell appears at or very near the periphery, for it is hardly reasonable that development from such a cell originating deep in the colony mass could ever manifest itself by wedge formation. To do so it would need to destroy or forcibly displace a great bulk of parent cells by an unusual degree of growth energy and cohesion, under conditions hardly favorable for active growth. The problem of the origin of the variant does not concern our present subject.

With these preliminary statements the question may be defined in terms of the relative growth rates of the parent and the variant, once it has appeared. Referring to this rate for the variant as G_v and for the parent as G_p we may form the ratio G_v/G_p which expresses the relative rates. This notation will be a convenience in the discussion to follow. It must be clearly understood that in applying the term "rate of growth" to a colony we mean something quite different from the same term as applied to a fluid culture. In a fluid, growth may be taken to refer to increase in cell mass or to increase in number of organisms. In the present sense, dealing with plate cultures, it means neither of these specifically but rather denotes an increase in the amount of surface occupied in unit time. Obviously, this depends in part upon both the multiplication rate and mass increase factors; but it is in addition highly dependent upon cell morphology and the presence of extracellular substances, both of which are commonly affected by variation.[4]

[4] It should be fairly clear that a form composed of short cells which separate completely and lie at all angles to one another will extend less rapidly than will a form composed of filamentous structures which tend to grow for some distance in a given direction. The end result in such cases would be for the short form to pile up in deep masses, whereas the filamentous form would develop to a thin

The ratio G_v/G_p may, of course, be equal to, less than, or greater than 1. Applied to wedges this has the connotations referred to below:

(A) If G_v/G_p is equal to 1, then the wedge will be straight sided and the sides will be the radii of the colony tangent to the initial cell. The angle will be governed by the distance of the apex from the center, being smaller as this distance is greater. When arising in colonies of macroscopic dimensions the wedge would be very narrow indeed and in the usual instance would not be detectable. This dependence upon the distance of the varying cell from the center of the colony can be readily seen by consideration of the simplified case of a colony composed of a single layer of cells in which growth is restricted to the marginal cells. If the variation takes place in this colony at a time when the periphery has N cells, then in the ideal instance the wedge will always include 1/N of the circumference. Obviously if the variation occurs at the first cell division the resulting wedge will include half the colony; if at the second division, it will include one quarter. The negligible character of any wedge (G_v/G_p equal to 1) occurring at the period of colony development when variants usually arise is demonstrated by postulating a 5 mm. colony of a coccus whose diameter is about 3 microns. If, at this stage, one cell in the periphery becomes a variant, the resulting wedge will under ideal conditions always include about $0.003/5\pi$ of the circumference. At the time when this colony has attained a size of 2 cm. the widest part of the wedge would be only about 0.01 mm., hardly a detectable portion.

(B) If G_v/G_p is less than one and the variation occurs later than the first few cell divisions, then no detectable wedge can be produced, for the variant area would be immediately surrounded and overgrown by the parent colony. We will have occasion to refer to this type again.

(C) If G_v/G_p is greater than 1, then a detectable wedge may form. Here the initial angle is governed both by the magnitude

veil covering a much larger area with a smaller mass of cell substance and a smaller number of cells. Similarly, extracellular substances of a semi-fluid nature can aid extension by their tendency to flow or by sheer bulk.

of G_v/G_p and by the distance of the varying cell from the center. Obviously in this case there are three possibilities: namely, G_v/G_p can be constant, increasing, or decreasing.[5] If the portion of the colonial circumference taken up by the variant wedge is to increase at a *constant* rate, the sides of the wedge must exhibit a certain degree of outward curvature. If it is to *increase* at an *increasing* rate, the curvature must be accentuated. If it is to increase at a *decreasing* rate, the curvature must be less than that

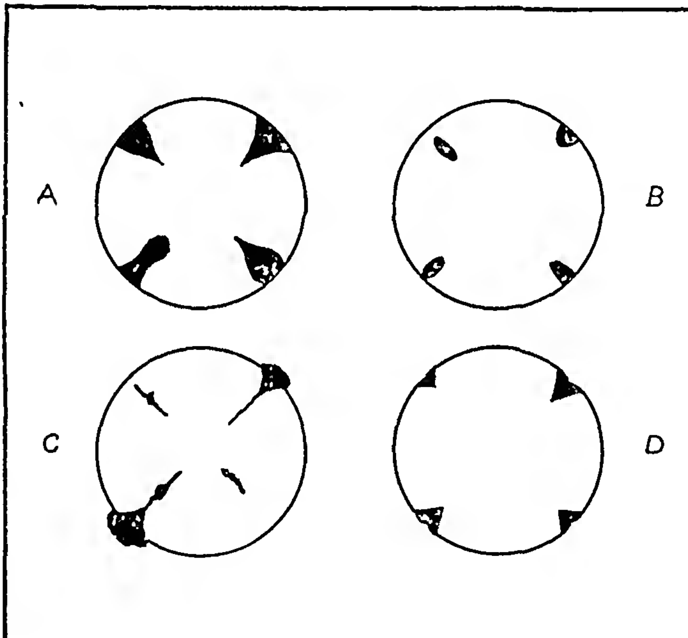

FIG. 2

for the constant rate and may show inward curvature. The wedge with straight sides would fall in this latter class. Frequently this rate of change in the ratio may alter with wedge growth, with a resulting change in degree or even direction of curvature. Thus, wedges of the type shown in figure 2a may arise. The form imparted to the wedge will be modified further

[5] That is, the rate of increase in the proportion of colonial periphery given over to the variant can be constant, increasing, or decreasing.

to some degree by the fact that whenever G_v/G_p is greater than 1 the wedge must extend slightly beyond the colony margin. This allows a slight lateral growth of the variant to take place, thus accentuating any tendency to outcurve. It appears from this that when G_v/G_p is greater than 1 the form of any variant wedge is a complicated problem in conflicting rates even when reduced to its simplest terms.

The outburst can now be defined as an example of G_v/G_p attaining a value much greater than is ordinarily the case. It is interesting to note that the outburst type of growth is marked by strong outcurvature of the sides and is, in general, confined to rough variants of the chain or filamentous type. This agrees with our previous statement regarding the spreading power inherent in this type of cell morphology. The island is then a result of G_v/G_p having a value greater than 1 but decreasing at a rate sufficiently great to impart a degree of inward curvature to the sides. In the course of such decrease, a critical point is reached when the sides become parallel to the radii of the colony, for at that point the ratio G_v/G_p becomes equal to 1 and, if the decrease proceeds beyond this point, the wedge will inevitably be surrounded, and an island will be formed (fig. 2b). This type of development accounts for the elongation mentioned earlier.

There is no reason for assuming that it is not possible for a carryover to take place through a reversal of this process. Assuming an initial variation with G_v/G_p equal to or only slightly greater than 1, then variant cells could develop within the growing parent colony as a very narrow wedge which might or might not be detectable. At a later time a marked increase in the value of G_v/G_p would result in an outburst (fig. 2c). Such a process is common in Friedländer's bacillus.

Secondary colonies could be produced in a similar manner. In all probability the variant cell which is overgrown when G_v/G_p is less than 1 continues to reproduce for some time as a buried nucleus in the parent colony mass. In some instances the growth from this variant may break through and appear on the surface when the G_v/G_p ratio becomes favorable at that point.[6]

[6] There would be, in fact, greater opportunity for the shift in ratio to take place in the central areas where secondary colonies usually arise than near the

It is highly probable that there is an additional mode of secondary colony formation. This is the production of variant cells, not in the actively growing margin, but in the older parts of the colony. In such an instance the restraining effect of the G_v/G_p ratio would still apply but with one modification, namely, that even with a favorable ratio, the only remaining degree of freedom is upward. Hence any variant produced under these conditions and having a ratio G_v/G_p greater or becoming greater than 1 must form a secondary colony. Secondary colonies arising from the germination of spores formed by the parent would be of this type. Rarely, secondary colonies may burrow downward and into the medium at the floor of the parent colony. When the parent is sufficiently transparent, secondary colonies with a ratio less than 1 may be observed as nuclei, although they may never emerge sufficiently to permit their being subcultured.

There exists one peculiarity of certain types of wedge which deserves mention as an illustration of a modifying influence that must be taken into account. In the S wedge appearing in M colonies of Friedländer's bacillus the margin of the wedge will usually be notched (fig. 2d). This indicates that the S growth immediately adjacent to the M growth is being stimulated so that in these regions G_v/G_p is greater than in the central portion of the wedge. Such stimulation by the proximity of other growth is by no means unknown and would help to clarify the fact that frequently the parent form shows a greater spreading rate than the variant when the two are studied as *isolated colonies*.

At times the dissociative process may become hyperactive with the result that a multitude of wedges may appear in the same colony at nearly the same time. If carried far enough, this can result in fusion and complete transformation of the border to the variant form. Similarly, the arising of many simultaneous secondary colonies can result in a ring of variant growth which may surround the parent when the dissociation is only slightly submarginal in origin. The effect of various modifications of the medium (pH, salts, sugars, etc.) in favoring or retarding colonial dissociation has received considerable study. As a rule the

growing margins, for it is here that the parent form will suffer most inhibition from its own products of metabolism.

assumption has been made that these factors acted through their influence on the production of the initial variant cell. In the light of the discussion in this paper it would seem plausible that this may not be the whole answer and that the effect of these modifying influences on the G_v/G_p ratio must be given careful consideration. Other environmental factors such as temperature, light and degree of aerobiosis should be accorded similar attention, for, as has been seen, a very small change in ratio could, under critical conditions, make all the difference between detectable and still-born variants. What answer would result from an experimental investigation from this point of view remains to be seen.

The analysis which has been presented here is primarily one of academic interest. Two deductions can, however, be made if the foregoing is correct.

1. There should exist a class of variants which we can hope to obtain only rarely and with difficulty by the method of selection from differentiated areas of dissociating colonies. These would be such forms as have a ratio G_v/G_p consistently less than 1. This class might, on the other hand, manifest itself readily under other growth conditions such as in fluid culture. Such variants should be unstable in the colonial state and should produce with relative frequency wedges of reversal forms.

2. It is well known that, as a result of encouraging colony dissociation, it is relatively easy to transform M forms to S and S forms to R, but that the reversal of this process by the same technique is frequently impossible. Furthermore, when it can be attained, the reversal is generally accomplished by secondary colony formation rather than by wedge formation. In liquid culture, on the other hand, where the organisms are dispersed, such reversals are obtained with relative ease even when they are impossible in colony development. The concept of relative growth rates as governing wedge formation would clarify this situation; for, if M dissociates to S and S dissociates to R with ease in the colony, we would need to assume G_s/G_m and G_r/G_s to be greater than 1 under these conditions. Then G_s/G_r and G_m/G_s would tend to be less than 1, and under similar conditions wedge formation would hardly be expected.

EFFECT OF CARCINOGENIC AND OTHER HYDROCARBONS ON THE GROWTH OF ESCHERICHIA COMMUNIOR

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In previous work from this laboratory (Goldstein, 1937) it was shown that methylcholanthrene and 1,2,5,6 dibenzanthracene accelerate the rate of reproduction of *Escherichia communior*, while phenanthrene does not. The following report deals with the microbiological test of these hydrocarbons, the details of preparation of their colloidal solutions, and the extension of the method to other compounds.

A survey of the recent literature indicates that the stimulating effect of carcinogenic compounds on the cellular activities of the lower organisms is being actively studied.

Using 1,2,5,6 dibenzanthracene and *Obelia geniculata* Reimann and Hammett (1935, a) reported that more new hydranths were formed in the test than in the controls. Of the several expressions of developmental growth, proliferation was accelerated and differentiation and organization tended to be enhanced. The same authors (1935, b) found new growth production from *anlagen* to be greater with methylcholanthrene than in controls. They conclude that the carcinogenic agents specifically stimulate the very earliest phases of growth.

The morphology and growth of yeasts are profoundly affected by methylcholanthrene according to Dodge and Dodge (1937) with the production of giant cells and increased differentiation of cells within the colony in cultures of *Saccharomyces ellipsoideus*

[1] Joint contribution no. 143 from the Biological Research Laboratories and no. 186 from the Research Laboratory of Organic Chemistry at the Massachusetts Institute of Technology.

Hansen. Total dry weight and fermentation were increased approximately one-third in saturated methylcholanthrene peptone glucose solution.

An increase in yeast proliferation of about 50 per cent was obtained by Cook, Hart, and Joly (1938) with 1,2,5,6 dibenzanthracene (9×10^{-4} molar) while anthracene in the same concentration had no effect.

Wright and Anderson (1938) apparently show that *water-soluble oxidation products* of 1,2,5,6 dibenzanthracene not only increased the weight of mycelium of *Fusarium lini* in a medium composed of mineral salts and glucose, but also gave a more rapid utilization of the glucose. The parent hydrocarbon under the same conditions was stated to have no effect on the growth of this microörganism.

EXPERIMENTAL PART

1. Preparation of hydrocarbons and colloidal solutions

All of the hydrocarbons employed in this study have been very carefully purified in order to strengthen the reliability of the bacteriological results; details concerning their purification are given below.

The method used for the preparation of aqueous colloidal solutions of the hydrocarbons was, in general, that of Boyland (1932). However, various modifications were necessary, and the exact conditions employed varied somewhat from compound to compound. It was found impossible to control all factors determining the stability of a solution, since solutions prepared as far as possible in the same way often varied greatly in their stabilities. One of the main factors affecting stability was the degree of purity of the hydrocarbon, impure preparations giving more unstable solutions. In all of the preparations the protective colloid used was gelatin (Difco Bacto), all of which was taken from the same lot in order to reduce the possibility of variations in bacteriological growth. Control solutions were prepared from distilled water and gelatin alone (0.5 gram per 100 ml.). The acetone (Merck's reagent) employed in making the colloidal

solutions was first dried thoroughly with anhydrous sodium sulfate and then distilled through a Davis fractionating column. Colloidal solutions of each hydrocarbon were prepared at two concentrations, 5 mgm. per 100 ml., and 30 mgm. per 100 ml. With anthracene and sym-triphenylbenzene, however, only the solutions of the lower concentration were stable enough for use. A detailed description is given for the preparation of anthracene solutions, whereas for the remaining compounds, only differences from this procedure are noted.

1. Anthracene. Anthracene (m.p. 213, Eastman Kodak Co.) was freed from the accompanying naphthacene by the method of chromatographic adsorption (Winterstein, 1934). Activated alumina, finely pulverized under dry petroleum ether, was used as the adsorbent. Anthracene (2 grams) was dissolved in one liter of ligroin (b.p. 70°–90°); the solution was then passed through an alumina-filled tower, 22 cm. long and 2 cm. in diameter. On concentration of the filtrate, white anthracene showing pronounced blue-violet fluorescence was obtained. After three recrystallizations from benzene, the product melted at 216°–216.5°.[2]

To a solution prepared from 0.5 gram gelatin and 100 ml. water, contained in a 400 ml. tall-form beaker, maintained at 60°, and rapidly stirred, was added through a burette (the tip of which was drawn out to a capillary) a solution of 5 mgm. anthracene in 15 ml. acetone. The addition required ten minutes, and care was taken that the stream of acetone did not touch the walls of the beaker, but that it was directed into the vortex created by the stirrer. To remove the acetone, the resultant colloidal solution was stirred and heated on the steam bath for three quarters of an hour, water being added from time to time to replace that lost by evaporation. The solution was then made up to 100 ml. The solution gave a slight precipitate after autoclaving.

2. Sym-triphenylbenzene. The substance was prepared according to the directions of Reddelien (1912). The crude product

[2] All melting points reported in this paper are corrected.

was purified by distillation *in vacuo;* after six recrystallizations from glacial acetic acid, the substance, dissolved in benzene, was shaken with dilute sodium hydroxide solution and water, dried, and recrystallized from benzene. M.p. 174.6°–174.9°.

The colloidal solution was prepared in a concentration of 5 mgm. per 100 ml. as described above for anthracene, except at a temperature of 50°. The solution was less stable than the anthracene solution.

3. 1,2,5,6 Dibenzanthracene. The yellow material supplied by the Eastman Kodak Co., and also prepared by the method of Clar (1929) was freed from the colored impurity, both by the use of chromatographic adsorption and also according to the method of Cook (1932). By both processes a white product was obtained, which, after five recrystallizations from benzene, melted at 262°–263°.

For preparation of a colloidal solution of concentration of 5 mgm. per 100 ml., 10 ml. acetone were employed, 25 ml. being used for a concentration of 30 mgm. per 100 ml. The preparation was carried out in both cases at 50°, and the acetone solutions could be merely poured into the rapidly stirred gelatin solution. The solutions of both concentrations were quite stable, only a very slight precipitate being formed on autoclaving.

4. Methylcholanthrene. The method of chromatographic adsorption did not diminish the yellow color of the product supplied by the Eastman Kodak Co. The product was purified through the picrate as described by Fieser and Seligman (1936). M.p. 179.8°–180.3°.

The colloidal solutions at both concentrations were prepared as in the case of dibenzanthracene, their stabilities being of the same order.

5. 1,2-Benzanthracene and Pyrene. These two substances were donated by Dr. E. B. Hershberg of Harvard University, to whom the authors are deeply grateful. Both substances were very pure, the benzanthracene melting at 160.5-161.5°, and the pyrene at 155-156°. Colloidal solutions were prepared at both concentrations as in the case of anthracene, except at temperatures of 50–55°. The solutions were very stable.

6. Phenanthrene. The Eastman product was vacuum sublimed and recrystallized ten times from ethyl alcohol. M.p. 99.4–99.8°.

Colloidal solutions at both concentrations were prepared as follows. To the gelatin solution at 75° was added the acetone solution of phenanthrene, as described for anthracene. The milky mixture was then boiled with vigorous stirring until all the acetone had been removed (one-half hour); it was then stirred for three hours, during which time the temperature was gradually lowered to that of the room. If the solution was allowed to cool down rapidly or without prolonged stirring, the phenanthrene soon precipitated out. Since phenanthrene is liquid at the boiling point of the solution and is hence very finely dispersed by the stirring, the solutions so prepared are very stable and give no precipitate after autoclaving.

2. *Bacteriological tests*

1. Organism. The organism used was a strain of *Escherichia communior* isolated at the Massachusetts Institute of Technology from feces in 1935. Its growth was excellent in the medium described below with the production of acid and gas in sucrose.

2. Medium. To make one liter, 5 grams each were taken of sodium chloride, sucrose, di-ammonium hydrogen phosphate, di-potassium hydrogen phosphate, and 2.43 milli-equivalents of HCl were added. This was tubed in 10 ml. amounts, plugged, and autoclaved at 15 lbs. for 20 minutes. Before autoclaving the pH was approximately 7.5; after autoclaving it was approximately 7.2.

For any given quantity of hydrocarbon solution the required amount of the salts was added and the medium was tubed and plugged in the usual manner. When autoclaving, however, the pressure was not allowed to exceed 8–10 lbs. and the time was ten minutes. Higher pressures and longer time intervals precipitated the hydrocarbon out of solution.

Although not all of the organisms were killed at the low pressure of autoclaving, resistant spore formers have not interfered with the test because the medium was inoculated with a heavy

suspension of the stock culture. In every experiment, one tube of hydrocarbon medium was kept uninoculated in the incubator for two reasons. One was to test the stability of the solution, and the other was to determine whether any growth occurred during the course of the experiment. The uninoculated tube did not show growth in any case, and the amount of precipitation of hydrocarbon varied. Only those experiments are reported in which the hydrocarbon solutions were relatively stable. Thus, for any test the only difference between the control and experimental media was the presence of the hydrocabron in the experimental tubes.

3. Inoculation and samples. After autoclaving, the tubes were cooled to 37°C. and each tube was inoculated with 0.1 ml. of a 24 hour culture in the medium used for daily transplants. All of the tubes were incubated at 37°C. and at desired time intervals duplicate tubes were removed. Their contents were pooled by mixing back and forth twice, and two ml. were taken with a volumetric pipette. The sample was placed in a sterile 8 oz. common narrow mouth bottle containing about 25 five-millimeter glass beads. After the addition of a sufficient amount of thionin blue in phenol to dilute the sample, a sterile rubber stopper was put in the bottle, and the sample was vigorously shaken to break up the clumps of organisms. Repeated samples were taken and counted as described below.

The dye solution consisted of a saturated solution of thionin blue (National Aniline Co. C.I. no. 920) in reagent alcohol diluted one to twenty in 1 per cent aqueous phenol. Thus to make one liter of dye solution take 950 ml. of distilled water containing ten grams of phenol and add 50 ml. of a saturated alcoholic solution of thionin blue.

4. Counting. A Petroff-Hausser counting chamber was used and any 200 squares were counted, using a 4 mm. 45× objective and a 15× ocular to get a total magnification of 675×. The cover glass was an optically plane glass 20×25 mm. and 0.18 mm. thick. A dilution of the culture which gives about six bacteria per square is advised by Glynn and his coworkers (1913–14), and it is suggested that one should allow 15 minutes to elapse

before making the count in order that the bacteria may settle. A total count is then made.

The counting chamber was previously washed with soap and water, then with reagent alcohol, and was rinsed and dried thoroughly before a sample was counted.

Counts of the numbers of organisms per square were made in order to determine their distribution in the counting chamber at various dilutions. About 6 to 7 bacteria per square gave a Poisson type of curve, indicating that the distribution was uniform at that dilution.

5. Graphs. Certain variables in the experimental procedure should be noted at the outset. The amount of growth of the test organism per unit time will vary with the amount of inoculum, and this varies from day to day. Consequently an absolute control growth curve to use as a basis of comparison for all of the hydrocarbons cannot be presented since the work was done over an extended period of time. Each control test was done on the same day as the experimental one and under identical conditions. The accompanying graphs of the total direct counts of the test organism summarize the results of experiments with the seven hydrocarbons mentioned above.

Graph 1 shows that the rate of reproduction of *E. communior* in the presence of 1,2 benzanthracene is practically the same as in a control solution. On the other hand, the accelerating action of 1,2,5,6 dibenzanthracene on growth in numbers is clearly shown in graph 2. The amounts inoculated into control and experimental cultures are almost identical, and the growth curves are alike until about the eighth hour. At this point the curves diverge and the difference in number of organisms in the hydrocarbon and control cultures increases with time. With solutions containing 5 mgm. per 100 cc. of dibenzanthracene, preliminary tests indicate that the growth may be increased, but that the difference occurs at a later period in the growth curve.

Similarly, methylcholanthrene increases the number of organisms as shown in graph 3. The amounts of inoculum in control and experimental cultures are reasonably alike, and the curves are parallel until about the ninth to tenth hour of growth, after

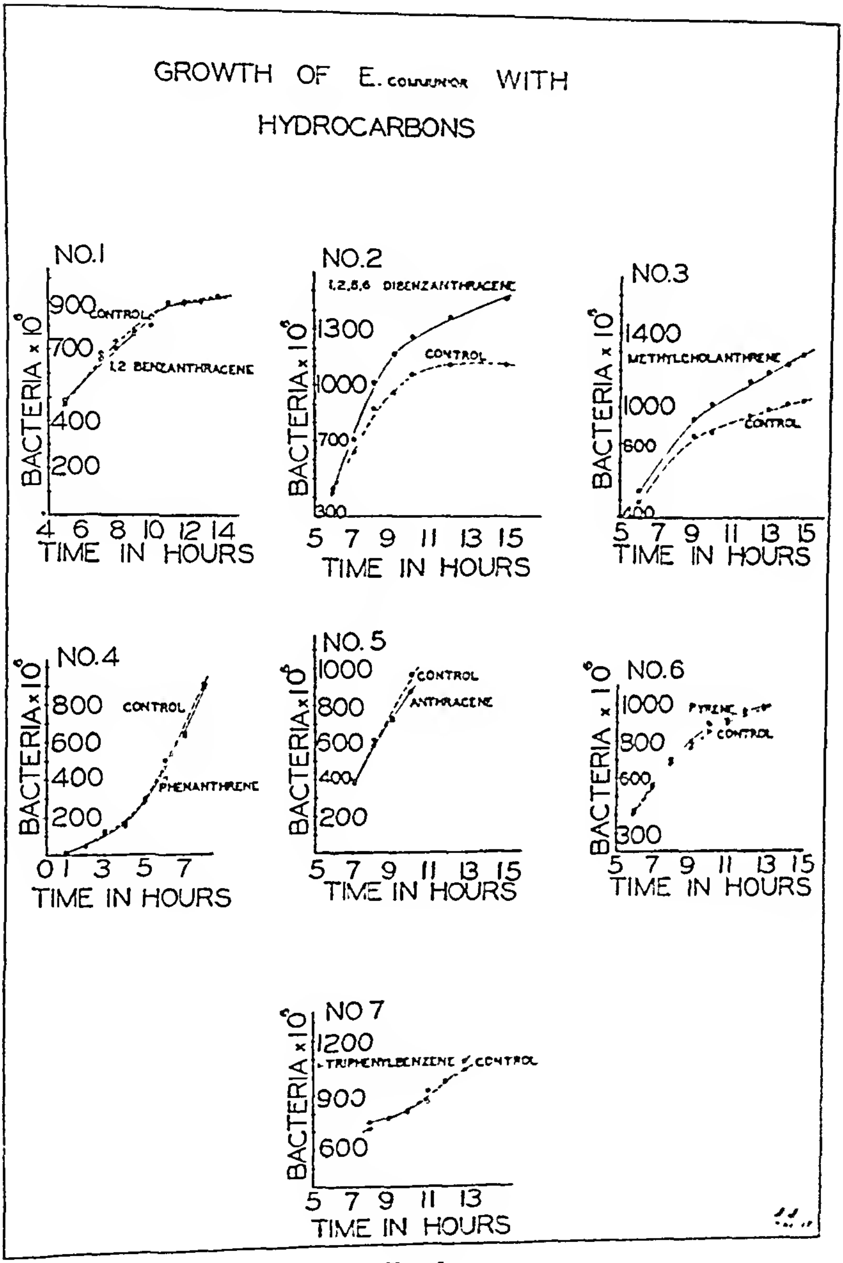
GROWTH OF E. communior WITH HYDROCARBONS
NO.1
CONTROL
1,2 BENZANTHRACENE
BACTERIA x 10^6
TIME IN HOURS
NO.2
1,2,5,6 DIBENZANTHRACENE
CONTROL
BACTERIA x 10^6
TIME IN HOURS
NO.3
METHYLCHOLANTHRENE
CONTROL
BACTERIA x 10^6
TIME IN HOURS
NO.4
CONTROL
PHENANTHRENE
BACTERIA x 10^6
TIME IN HOURS
NO. 5
CONTROL
ANTHRACENE
BACTERIA x 10^6
TIME IN HOURS
NO.6
PYRENE
CONTROL
BACTERIA x 10^6
TIME IN HOURS
NO 7
TRIPHENYLBENZENE
CONTROL
BACTERIA x 10^6
TIME IN HOURS

Fig. 1

which the curves diverge. The magnitude of increase in growth is comparable to that shown in the preceding graph.

Graph 4 illustrates the absence of any effect on growth in numbers with phenanthrene. The control and experimental curves are smooth and may be considered as identical. Repeated tests with phenanthrene at later hours of growth than are shown in the diagram gave the same results.

Anthracene has no effect on growth, as shown in graph 5. The hydrocarbon solution in this instance had 5 mgm. per 100 ml. of anthracene. Higher concentrations gave unstable solutions. The growth curve for pyrene in graph 6 is identical with the control. In this case the hydrocarbon solution contained only 5 mgm. per 100 ml., but higher concentrations were also used, and these gave the same results. Finally, graph 7 shows the absence of any effect with sym-triphenylbenzene which was used in the same concentration as anthracene.

DISCUSSION AND SUMMARY

The differences between the activities of single cells and tissue cells are well recognized, and consequently these investigations were begun with recognition of the desirability of rigorously testing the hypothesis that compounds which are carcinogenic in animal tissues might also cause single cells to reproduce quickly. The experimental evidence indicates a high degree of correlation between carcinogenicity of compounds and stimulation of bacterial reproduction.

That the increased numbers of organisms observed is not due to too large an inoculum is clearly evident from graph 2, for example, where the amounts of inoculum in control and experimental cultures are almost exactly the same. The amount of increase in the stimulated cultures is not quantitative in the sense of being absolute, but in repeated tests, the results are highly comparable on a qualitative basis.

The mechanism by which the carcinogenic compounds accelerate the growth of the test organism is quite obscure at present. Further data must be obtained on some of the more important factors involved in the reactions. Thus the amount

of sugar utilized per unit time should be studied, as well as the action of dehydrogenases, the importance of aerobic versus anaerobic conditions, and the kinds and amounts of products formed.

It may be of interest to note that in the experiments with 1, 2, 5, 6 dibenzanthracene and methylcholanthrene a large amount of gas was observed at about the eighth to tenth hours of growth in the experimental tubes. There was little or no gas in control cultures at the same stage of growth.

CONCLUSIONS

1. An increase in the numbers of cells of *Escherichia communior* can be induced by the carcinogenic compounds 1, 2, 5, 6 dibenzanthracene and methylcholanthrene in a concentration of 0.3 milligram per ml. respectively.

2. Under the conditions of the experiment the number of organisms in cultures of *Escherichia communior* is not increased by any of the following compounds: 1, 2, benzanthracene, phenanthrene, anthracene, pyrene, and sym-triphenylbenzene.

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ADAPTATION OF THE PROPIONIC-ACID BACTERIA TO VITAMIN B_1 SYNTHESIS INCLUDING A METHOD OF ASSAY

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In a paper dealing with the nutrition of the propionic-acid bacteria Wood, Andersen, and Werkman (1938) report that one species *Propionibacterium pentosaceum* (49W), can be "trained" to grow as well in the absence of vitamin B_1 as in its presence. Their data show that adaptation occurred after continuous serial transfer in an ammonium sulfate medium containing only ether extract of yeast extract as a stimulant; the organism gradually acquired the ability to dispense with vitamin B_1. They did not determine whether the organism, "trained" to dispense with the vitamin, acquires the ability to synthesize it, or whether its metabolism is diverted so that the vitamin is no longer required. The present study was undertaken to determine which of the two possibilities occurs. It has been found that the organism acquires the ability to synthesize vitamin B_1. A bacteriological method of assay of vitamin B_1 employed in the study is also described.

EXPERIMENTAL

1. *Method of assay*

The present authors (1938) have shown previously that the addition of vitamin B_1 to cells of *P. pentosaceum* which had been grown in the basal medium of Tatum, Wood and Peterson (1936) in the absence of the vitamin, resulted in an almost immediate increase in their anaerobic pyruvate metabolism as judged by the evolution of CO_2. The assay to be described is based on the application of the above observation.

The stock culture of *P. pentosaceum* was grown on a medium consisting of the following: Glucose, 1 per cent; Yeast extract (Bacto), 0.3 per cent; Peptone (Bacto), 0.2 per cent. It was transferred daily, 4 drops of inoculum being transferred to 25 ml. of the above medium. The incubation temperature was 30°C.

The basal medium of Tatum, Wood and Peterson (1936) was employed together with cystine and hydrolyzed casein as follows: Glucose, 1 per cent; Sodium acetate, 0.6 per cent; Ammonium sulfate, 0.3 per cent; Cystine, 0.0050 per cent; Hydrolyzed casein, 0.075 per cent; Ether extract of 3 grams yeast extract per 100 ml., Speakman's salts in half concentration.

Three hundred ml. of the above were placed in 500 ml. flasks and also 25 ml. and 5 ml. portions tubed.

In preparation for a vitamin B_1 assay the actively growing cells of *P. pentosaceum* from 25 ml. of the yeast-extract peptone medium were aseptically recovered by centrifugation, washed twice with two 10 cc. portions of sterile distilled water and resuspended in 5 cc. of sterile water. One drop of this suspension was used to inoculate a 5 ml. portion of the basal medium of Tatum *et al.* (1936). This culture was incubated 24 hours at 30°C. and at the end of that time the centrifuged cells washed with 10 ml. of sterile water, resuspended in 5 ml., and the entire contents used to inoculate a 25 ml. portion of the basal medium. This in turn was incubated for 24 hours at 30°C. and, after washing in 10 ml. of sterile water, was employed as an inoculum for 300 ml. of the basal medium. The contents of the flask were incubated for 72 hours at 30°C., the cells obtained were washed twice in distilled water, and then employed as test organism for a manometric assay of vitamin B_1. The second washing was carried out in a 12 ml. graduated centrifuge cup and the volume of cell paste was recorded after 20 minutes centrifuging at about 1900 r.p.m. Cells of *P. pentosaceum* can be readily recovered by centrifugation.

The cell paste obtained from a single flask of medium was made up to 12 ml. with M/15 phosphate (pH 5.6) and 1 ml. of this suspension was placed in the main vessel of each Warburg cup. Nine milligrams of pyruvic acid as sodium pyruvate were

employed as a substrate and placed in the side arm. Varying amounts of vitamin B_1 contained in a volume of 0.1 ml. of water were added to the cells in the main vessel. After shaking in the water bath at 30°C. for 30 minutes, the contents of the side cup were tipped into the main vessel and readings taken. The atmosphere was nitrogen, from which any oxygen had been removed by passage over hot copper. The total volume contained in the cups was in all cases 2.3 ml. Simple Warburg manometers

TABLE 1

Effect of varying vitamin B_1 concentration on anaerobic pyruvate metabolism of P. pentosaccum

EXPERIMENT NUMBER	YIELD OF CELL PASTE	GAMMAS VITAMIN B_1 ADDED								
		0	0.25	0.125	0.0625	0.0312	0.0156	.0078	.0039	.002
		CO_2 evolved, mm^3								
Part 1										
1	0.50	42	366	338	305	157	113	85	57	56
2	0.50	40	375	333	253	198	111	84	65	53
3	0.48	48	351	316	263	176	123	98	69	59
4	0.48	46	353	296	249	199	102	98	76	63
5	0.49	43	354	314	247	193	122	98	72	62
6	0.50	40	348	317	253	169	147	84	66	56
7	0.45	58	319	287	235	171	107	81	79	64
Part 2										
11	0.54	50	292	254	217	135	105	84	74	66
12	0.50	55	300	265	238	146	114	87	66	66
13	0.51	68	300	278	235	147	114	104	57	51
14	0.52	69	293	273	246	145	110	90	62	62
15	0.49	46	297	269	211	146	104	84	79	65
16	0.50	40	322	290	249	166	112			

were employed and the details of the manometric technique used is described by Dixon (1934).

Results of several runs at various times are shown in table 1. The cells employed in part 1 of the table were grown in a medium containing ether extract of yeast prepared separately from that used in part 2. The results shown were obtained at the conclusion of a $2\frac{1}{2}$ hour run and are corrected for endogenous CO_2 production.

The results show that the response of cells grown in the manner described to the addition of varying quantities of synthetic vitamin B_1[1] is sufficiently consistent to warrant its use in the assay of the vitamin. A fairly accurate approximation of the vitamin content of a material may be obtained if extracts are tested in several dilutions with their vitamin content within the range of the test described.

The manometric determination described is affected neither by the presence of the pyrimidine[2,3] or thiazole[3,4] fraction of vitamin B_1 nor by nicotinic amide in the amounts tested (up to 10 gammas).

As two advantages of this method over animal assays may be mentioned, the short period actually involved in the assay (about 4 hours including $2\frac{1}{2}$ for the manometric run) and the small amount of material required for a single assay, 0.025 to 0.05 grams of dried bacterial cells being sufficient for an assay.

Other methods employing microorganisms which have been described in the literature are the growth tests of West and Wilson (1938) who used *Staphylococcus aureus* and of Schopfer and Jung (1936) who used the mold, *Phycomyces blakesleeanus*; a fermentation test with yeast as the test organism has been described by Schultz, Atkin and Frey (1937).

2. Adaptation of P. pentosaceum to synthesis of vitamin B_1

Cultures of *P. pentosaceum* which had been carried on the basal medium, described earlier, for (1) only a few transfers and (2) until they had been "trained" were compared as to their vitamin B_1 content and their activity on pyruvate. It has already been shown (Silverman and Werkman, 1938) that the vitamin is essential in the anaerobic pyruvate metabolism of this group of organisms.

A transfer of the organism was made from the yeast extract-peptone medium after washing twice, to the basal medium of

[1] Kindly supplied by the Winthrop Chemical Company

[2] Pyrimidine fraction = 2 methyl 5 bromoethyl 6 aminopyrimidine hydrobromide.

[3] Thiazole fraction = 4 methyl 5 β hydroxyethylthiazole

[4] *Kindly supplied by Merck and Company.*

Tatum, *et al.* (1936). It was carried through eight transfers, using only a single drop as inoculum, into 5 ml. portions of medium; in the ninth transfer the cell contents of the 5 ml. portion were used to inoculate 25 ml. of the basal medium; and in the tenth transfer, the entire cell contents of the 25 ml. portion were used to inoculate 300 ml. of medium. The cells were not washed during the transfers on the basal medium.

In a manner similar to the above, a culture taken from a yeast-extract peptone medium was carried through three transfers on the basal medium—from 5 ml. to 25 ml. to 300 ml. Here, and in the above, transfers were made at 24-hour intervals and the 300 ml. portions were incubated for 72 hours. Hereafter, the cells carried ten transfers on the basal medium lacking vitamin B_1 will be referred to as "trained", those carried only three transfers, as "untrained." It was found that cells of *P. pentosaceum* after passing through three transfers show a marked depletion of vitamin B_1; however, by the tenth transfer the ability to synthesize the vitamin is definitely manifested.

In comparing the yield of cell material from 300 ml. of medium we find that we obtain twice the volume of the "trained" cells in comparison to the "untrained." Wood, Andersen and Werkman (1938) have already indicated the increase in acid production from glucose as the organism becomes adapted.

Both the "trained" and "untrained" cells were assayed for vitamin B_1 content. Extracts from cells which had been dried in a vacuum desiccator at room temperature for 24 hours were prepared by suspending 0.1 gram of dried cells in 5 ml. of water, the suspension kept in a boiling water bath for 5 minutes, and then clarified by centrifuging. The supernatant fluid was then assayed for vitamin B_1. By taking the average value found for several dilutions of both the "trained" and "untrained" cells we find the "trained" cells have a vitamin B_1 content of 6.25 gammas per gram of dry cells and the "untrained" cells contain but 0.40 gamma per gram of dry cells. The extraction of the vitamin with water was, of course, incomplete; but aqueous extraction should remove comparative amounts of the vitamin and so justify the comparisons made.

Since such a large difference in vitamin B_1 content of the

"trained" and "untrained" cells exists we should expect a corresponding difference in the pyruvate metabolism of the cells, inasmuch as the vitamin is essential in biological pyruvate breakdown. This assumption is borne out in figure 1. The "untrained" cells show almost no activity on sodium pyruvate: the "trained" cells show almost a maximum anaerobic pyruvate

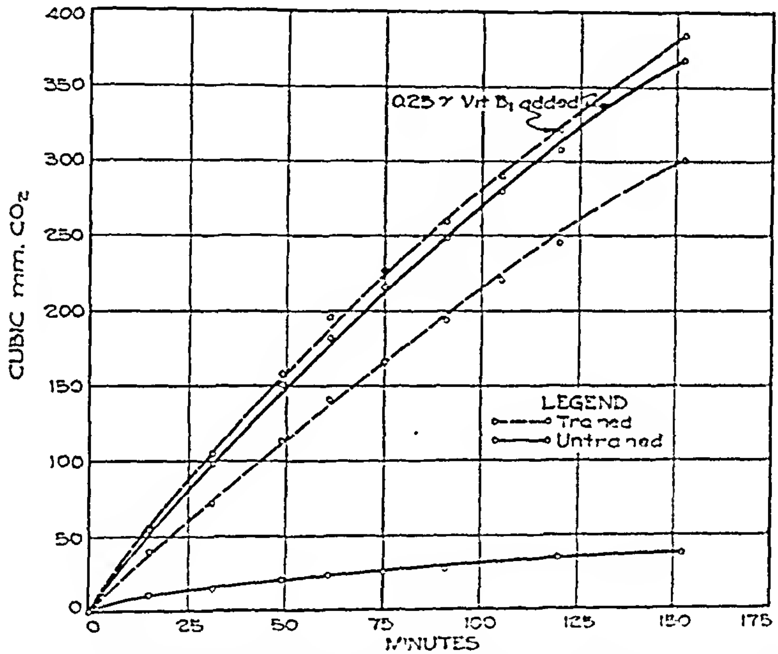

FIG. 1. COMPARISON OF THE ANAEROBIC PYRUVATE METABOLISM OF "TRAINED" AND "UNTRAINED" CELLS OF P. PENTOSACEUM

Cells, 1 ml. of 1:20 suspension (by vol.) in M/15 phosphate, pH 5.6. Substrate, 9 mgm. pyruvic acid. Atmosphere, nitrogen. Temperature, 30 C. Total volume, 2.3 ml. Vitamin B_1 added directly to cell suspension.

metabolism judging from the slight increase on addition of 0.25 gamma vitamin B_1. The addition of 0.25 gamma of the vitamin to the "untrained" cells increases their metabolic rate to substantially that of the "trained" cell suspension containing added vitamin B_1; indicating that at least so far as the anaerobic pyruvate metabolism is concerned. the "trained" cells differ from the "untrained" only by having a higher vitamin B_1 content.

Table 2 recapitulates the differences found in a single series of experiments in which the "trained" and "untrained" cells were compared.

The finding that *P. pentosaceum* can be trained to synthesize vitamin B_1 is somewhat similar to the report of West and Wilson (1938a) that *Rhizobium trifolii* in a purified basal medium can also synthesize the vitamin in quantities to permit normal growth; however, this apparently is not a case of adaptation; for to initiate growth, a trace of the vitamin is required, after which the cells bring about its synthesis.

The mechanism of adaptation may be that of mutation and subsequent selection, or the development of the vitamin B_1 synthesizing enzyme system within the original cell, or perhaps, the utilization of a latent mechanism which was always present in the cell, but employed only under stress, i.e., in the continued absence of vitamin B_1. Which mechanism it is, however, cannot be stated at the present time.

TABLE 2

Comparative yields of cells, their vitamin B_1 content and anaerobic pyruvate metabolism

	YIELD OF WET CELLS	VITAMIN B_1 CONTENT PER GRAM DRY CELLS	ANAEROBIC PYRUVATE METABOLISM
	ml.	*gamma*	*mm³ CO_2/hr.*
"Trained" cells	1.15	6.25	120.3
"Untrained" cells	0.50	0.40	15.2
Ratio $\frac{\text{"trained"}}{\text{"untrained"}}$	2.3	15.6	7.9

SUMMARY

1. A technique for the assay of vitamin B_1 is described which is based on the increased anaerobic pyruvate metabolism of vitamin B_1-deficient cells of *Propionibacterium pentosaceum* occurring on the addition of the vitamin.

2. *Propionibacterium pentosaceum* if transferred continuously on a basal medium free of vitamin B_1 can be "trained" to synthesize this vitamin to satisfy its growth requirements.

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PRESERVATION OF BIOLOGICAL FLUIDS (BACTERIOPHAGE, VACCINES AND VENOM SOLUTIONS) WITH ALKYL-DIMETHYL-BENZYL-AMMONIUM-CHLORIDE*

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The general properties of this compound have been evaluated rather extensively in other reports. The reported phenol coefficients are as follows: 150 at 20°C. (Maier and Müller, 1936), 279 at 20°C. (Dunn, 1937), and 275 at 37°C. (Heineman, 1937), respectively for *Staphylococcus aureus* no. 209 Dept. of Agriculture and 200 at 20°C. (Maier and Müller, 1936), 250 at 20°C. (Dunn, 1937), and 176 at 20°C. (Heineman, 1937), for *Eberthella typhosa* (Rosenbach) Dept. of Agriculture at the same temperature tested by the F. D. A. method.

Domagk (1935) shows that an aqueous solution of this compound in a concentration of 1:1,000 can be given by mouth to guinea pigs as their only source of fluid for months without apparent deleterious effect upon their health. These toxicity studies indicate that rabbits tolerate 1.2 ml. per kilogram of body weight of a 10 per cent solution of the compound, when given intraperitoneally or subcutaneously. A solution ranging in concentration from 1:2,500–4,000 appears to be safe for use in the eye as it was shown to produce little or no reaction, when applied repeatedly to the conjunctiva of albino rabbits (fig. 1). To demonstrate this, the procedure was as follows:

Of the solution 1:4,000, one half ml. was instilled into the conjunctiva of the rabbit and kept there for one minute. Immediately after, the eye was washed out with saline. For comparison we used a $3\frac{1}{2}$ per cent concentration of tincture of iodine

* Trade name Zephiran.

which was administered under the same conditions to a second animal. Only one application was necessary to produce the described effect with the tincture. Severe reactions resulted. Within three weeks the animal treated with the tincture of iodine showed a completely opaque cornea and severe inflammation with discharge of pus existed during most of the period. The animals which were treated with the ammonium chloride com-

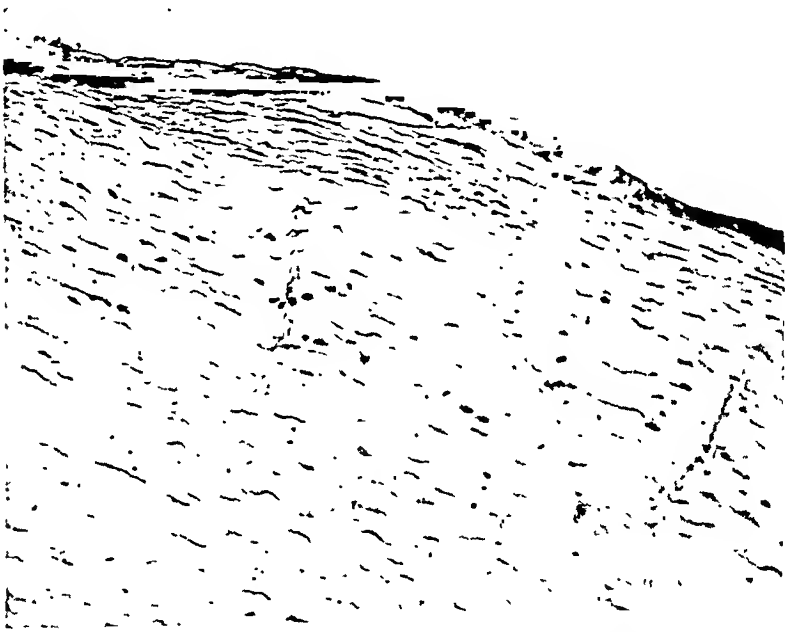


FIG. 1. CROSS SECTION THROUGH CORNEA OF RABBIT

Alkyl-dimethyl-benzyl-ammoniumchloride 1:3,000 was applied daily for ten days to this eye. There is little if any deviation from the aspect of the normal untreated cornea.

histological examination confirmed the gross anatomical findings, namely, no injury to or thickening of the anterior epithelium in the case of the ammonium chloride, enormous thickening, fibrotic changes and scar tissue formation extending through the cornea into the *substantia propria* in the animal treated with tincture of iodine (fig. 2).

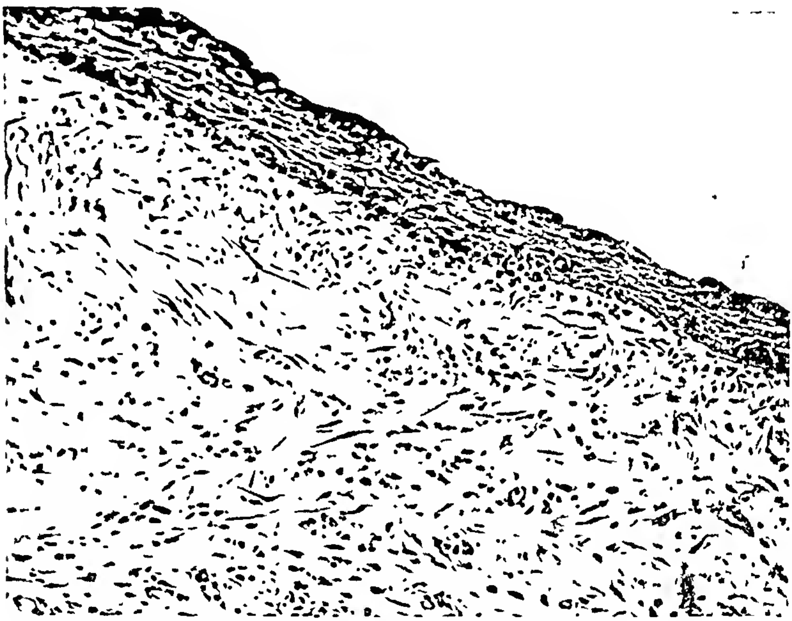


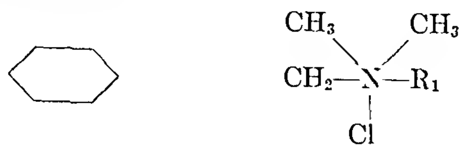
FIG. 2. CROSS SECTION THROUGH CORNEA OF RABBIT TREATED WITH TINCTURE OF IODINE $3\frac{1}{2}$ PER CENT

One application of tincture of iodine $3\frac{1}{2}$ per cent was sufficient to produce these profound changes in the cornea. There is enormous thickening, fibrotic changes and scar tissue formation extending through the cornea into the substantia propria.

From such studies as these it appeared probable that the absence of mercury and phenol radicals and the low toxicity of this compound might make it very useful for the preservation of biological fluids. In the following, results obtained in treating bacteriophage, vaccines and venom solutions with the compound are recorded.

STAPHYLOCOCCAL BACTERIOPHAGE

We began with the determination of the germicidal range of alkyl-dimethyl-benzyl-ammoniumchloride. The chemical structure of the compound is:



The alkyl residue (R_1) is composed of the radicals C_8H_{17}, $C_{10}H_{21}$, $C_{12}H_{25}$, $C_{14}H_{29}$, $C_{16}H_{33}$, and $C_{18}H_{37}$, their source being the mixture of fatty acids of cocoanut oil in original proportion. The product is soluble in water, acetone and alcohol.

Staphylococcus aureus (Dept. of Agriculture no. 209) was killed within and below 24 hours by concentration of the ammonium chloride of 1:80,000 to 1:100,000. Tests show that at 30°C. these organisms are killed within 10 minutes by concentrations of the compound between 1:30,000–35,000. The range of inhibition we found to be very large, varying from 1:100,000 to 1:800,000. At a concentration of 1:800,000 in the original tube after a contact of 24 hours at room temperature, the upper limit of bacteriostatic activity has therefore been reached, both the original and the subculture tubes showing growth.

Test tubes containing 10 ml. of bouillon (Difco nutrient) were inoculated with 0.5 ml. of a 24 hour broth suspension of *Staphylococcus aureus* (Dept. of Agriculture no. 209). The broth contained the disinfectant in solution in concentrations ranging from 1:40,000 to 1:800,000. Within 24 hours the tubes were opened again and subcultures were made. Table 1 shows the results obtained.

From table 1 it will be noticed that the germicidal range reaches up to 1:100,000, the bacteriostatic range from there up to 1:800,000, and the inactive range from there on. Marshall and Hrenoff (1937) propose the term "disinfectant spectrum" for the relative evaluation of disinfectants whose bactericidal, bacteriostatic and inactive concentrations have thus been determined.

On the basis of these results, it was decided to choose a dilution

of 1:50,000 of the disinfectant in staphylococcal bacteriophage. The following scheme was used in these tests:

1. 10 ml. bouillon plus 0.1 ml. of a 24-hour culture. Growth control tube.
2. 10 ml. bouillon plus 0.5 ml. phage disinfectant mixture.
3. Same as 2. (Tube three is used in addition to no. 2 tube, as there might be in tube 2 or 3 an initial lysis with a secondary growth following a short time later. In case there is initial lysis in tube 2 or 3, one of the tubes 2 or 3 can be filtered and the resulting phage propagated separately, regardless of the outcome in the other tube.)
4. Sterility Control of Phage.

TABLE 1

Test organisms: Staphylococcus aureus. Incubation temperature 37°C. Time of contact 24 hours

RANGE	CONCENTRATION	ORIGINAL TUBES	SUBCULTURE TUBES
Germicidal	1:40,000	–	–
	1:60,000	–	–
	1:80,000	–	–
	1:100,000	–	+
Inhibition	1:200,000	–	+
	1:300,000	–	+
	1:400,000	–	+
	1:500,000	–	+
	1:600,000	–	+
	1:700,000	–	+
	1:800,000	+	+
Inactive			

– no growth.
\+ growth.

After phage lysis had taken place, filtration by Berkefeld N followed. The phage obtained was kept in the refrigerator and tested at intervals and after preservation with the compound in proportion of 1:50,000. After intervals of two and three weeks, checks were made, to insure the viability of the phage. The longest period so far checked was three months, in which interval

the phage had not lost any of its lytic power in the presence of the alkyl-dimethyl-benzyl-ammoniumchloride at the concentration under test.

VACCINES

In the preparation of autogenous vaccines, alkyl-dimethyl-benzyl-ammoniumchloride was added to the vaccine to make a final concentration of 1:50,000. This concentration was agreed upon as previous tests had shown that the germicidal range was as high as 1:100,000.

VENOM SOLUTIONS

It has been stated (Macht, 1936) that rattle-snake venom solutions (*Crotalus adamanteus*) are very unstable, as they are affected by heat and rapidly decompose even at room temperature. To unaltered venom solutions prepared from dried venom (the venom was obtained freshly and frozen and dried rapidly *in vacuo* and over sulphuric acid), was added the ammonium chloride compound to make a final concentration of 1:50,000. In the same way, we treated venom solutions of the cotton-mouth moccasin (*Ancistrodon mocasen*). Over a period of 6 months we have not been able to demonstrate loss of toxicity, when tested in mice. Fresh venoms tested by intraperitoneal injection of the venom gave a minimum lethal dosage of 40–50 gamma for the rattle snake and 150 gamma for the moccasin venom in mice averaging 20 grams. However, such venoms when sterilized by filtration through Berkefeld N filters did show an appreciable loss in toxicity, as determined in the mouse test, a loss which probably is due to absorption by the filter. To venom solutions the compound was added in the same concentration, with no apparent change or precipitation of protein material.

DISCUSSION

As to the significance of such studies, recent investigations, (Bronfenbrenner, 1928), have shown that in many respects the behavior of the phage toward chemicals is not essentially different from that of enzymes on one hand and from that of viruses on the other. Prausnitz and Firle (1924) mention that the

adaptation of phage to phenol and mercuric chloride was necessary. In these studies no perceptible degree of adaptation was necessary, to produce bacteriophage in the presence of alkyl-dimethyl-benzyl-ammoniumchloride.

CONCLUSIONS

A new compound, alkyl-dimethyl-benzyl-ammoniumchloride has been studied in reference to its germicidal, bacteriostatic and inactive zones in accordance with the term "disinfectant spectrum" as proposed by Marshall and Hrenoff (1937). The effect of this compound in dilutions of 1:4,000 has been studied on the cornea of the rabbit by histological methods. Comparisons were made with $3\frac{1}{2}$ per cent tincture of iodine. It was shown that one single application of the iodine tincture was sufficient to destroy the cornea completely and produce permanent opaqueness in the same. The compound under discussion has been applied to the cornea daily for ten days with no consequences.

A dilution of 1:50,000 in staphylococcal bacteriophage was found not to interfere with the reproduction of the phage. Furthermore, the phage-disinfectant mixture was found to be capable of reproduction of new phage after 3 months contact in the refrigerator. The same concentration of the compound was employed in the preservation of vaccines and venom solutions.

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BACTERIAL VARIATION: FORMATION AND FATE OF CERTAIN VARIANT CELLS OF BACILLUS MEGATHERIUM

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At present there is no general agreement among bacteriologists concerning the functional significance of the numerous, and often striking, cellular variants which can under certain circumstances be found in many bacterial cultures. Widely divergent hypotheses have been advanced to account for their existence, but no entirely convincing body of evidence supporting any one theory is available. In the investigation reported here the problem was approached from the following viewpoints: (1) the appearance and development of so-called "normal" cells in favorable environments, (2) the manner of formation of variant cells, (3) the destiny of variant cells in the environment in which they arise, (4) the conditions leading to their production, and (5) their behavior when transferred to new and favorable surroundings.

Bacillus megatherium, strain N. Y. U., was employed throughout the investigation, because it shows, under appropriate cultural conditions, so many of the cellular and colonial variants which have been described in the literature for various bacterial species. Furthermore, it is one of the largest of the common bacteria, and therefore lends itself admirably to intensive microscopic study.

MEDIUM AND METHODS

Plain nutrient broth containing 0.3 per cent Bacto-meat-extract and 1 per cent Bacto-peptone and having a final pH of 6.8 to 7.0

[1] This paper covers part of a dissertation submitted by the Senior author (Now Instructor in Bacteriology, Albertus Magnus College, New Haven, Conn.) to the Graduate School of Yale University in partial fulfilment of requirements for the degree of Doctor of Philosophy.

was used as the basal medium throughout this investigation. When a solid medium was required 1.5 per cent Bacto-agar was added to this fluid base. In some instances the nutrient concentration in the medium was varied, as will be seen later in this paper.

The observations reported here were made on micro cultures, the hanging block method of Hill, as modified by Rettger and Gillespie (1935), being used for the purpose. The cultures as a rule were incubated at 30°C. During long-continued microscopic observation the blocks were held at ordinary room temperature, since slight variations in temperature did not appear to affect the results materially. Serial photomicrographs were made during the course of the experiments. A Bausch and Lomb vertical type K camera and Wratten and Wainwright metallographic plates were employed.

APPEARANCE AND DEVELOPMENT OF BACILLUS MEGATHERIUM CELLS IN FAVORABLE ENVIRONMENTS

Three cell types were observed in young cultures of *Bacillus megatherium*. Two of these types, "normal" rods and long filaments, were also often seen at the peripheries of well-isolated colonies when the colonies were many days old. It may be assumed, however, that such cells were still developing in a favorable environment, because living conditions continue to be favorable at colony peripheries for varying periods of time, depending on the amount of unpopulated medium surrounding the colonies. "Normal" rods and long filaments are, in reality, not sharply defined types; they differ from each other only in cell length. All cell-lengths intermediate between the two extremes were observed at one time or another in young cultures. Although the exact conditions which led to the formation of long filaments, instead of shorter filaments or long rods, were not determined, there were indications that the relative favorableness of the environment was an important factor. During the first few hours of growth on fresh, moist, rich media *B. megatherium* always formed extremely long filaments, whereas on drier and poorer media the cells were comparatively short.

Distinctly favorable conditions appeared to stimulate cell growth markedly, whereas they had only a slight accelerating influence on cell division. Consequently, cells often became extremely long filaments before division took place. The operation of the same principle was reported by Rettger and Gillespie (1935). They observed that cell growth was influenced more by unfavorable conditions than was cell reproduction. Cells continued to divide after elongation had practically ceased, and coccoid organisms were often produced as a result. According to both the earlier and the present observations, coccoid cells and long filaments are the two extremes of variations in cell length. In other words, cell length appears to be a measure of balance between the growth rate and the rate of reproduction.

A third cell type, the "round end balls" reported by Rettger and Gillespie in 1935 was also frequently found in young vigorously-growing cultures. This type of cell appeared to reproduce very slowly if at all, and when present was soon hidden from view by the rapidly developing rods or filaments in the colony. It was seldom seen again in material taken from the colonies after aging, having apparently disintegrated.

THE FORMATION OF FILAMENT BRAIDS OR TWISTS

In colonies which were composed largely of long filaments a peculiar type of "braiding" or "twisting" cells frequently made its appearance. Under certain circumstances many groups of "twisted" cells appeared around the peripheries of young and actively-growing colonies. They developed not only on hanging blocks, but also in slant agar cultures and on agar plates. Occasionally, two or three braided pairs wound themselves together to form a thick rope-twist. Pairs of cells were always involved, braids made up of three or five filaments never being observed. Intertwining cells were ordinarily strongly Gram-positive. They were, furthermore, not stained by Congo-red. Similar forms have been reported by Jay (1928) and others. Miessner (1931) published a photograph which shows twisted cells in a culture of *Clostridium novyi.*

Because the appearance of entwined filaments suggested con-

jugating cells, the manner of their formation was studied further. Figures 19 through 23 are photomicrographs selected from a series of fourteen which show different stages in the formation of a single braided pair. A bent filament (fig. 19) divided in such a way that one member of the pair of daughter cells was longer than the other. The tips of the two new cells remained attached to each other after division by a tough but invisible connecting thread. Such threads have been shown to occur between *B. megatherium* cells, and have been referred to as "protoplasmic strands" or "plasmodesmids." Attempts to break up chains of *B. megatherium* cells with micro-needles indicated that the connecting strands are frequently very tough and strong.

As the longer, and possibly more rapidly-growing, member of the attached pair lengthened it approached the other at a slight angle and pushed it over and over, causing it to rotate on its long axis (figs. 20 and 21), and, so long as the two growing cells remained attached, the twisting force of the longer continued to be effective. As soon as the link connecting the two cells broke, the intertwining ceased, and the freed tip of each organism grew out as an independent individual (fig. 23). As twisted filaments become older they segment in the usual manner. The twisting phenomenon described here appears to be dependent upon mechanical or physical factors, rather than upon physiological affinities between cells.

THE FORMATION OF BRANCHED, SWOLLEN, POINTED AND GLOBULAR CELL TYPES

So long as cultural conditions were conducive to rapid growth and reproduction, colonies were made up almost exclusively of rods of varying length, or of filaments. It was only after growth and reproduction had almost ceased that sporulation or variant cell formation, or both, occurred. The production of the various variant cell types was followed closely, and recorded by serial photomicrographs or camera-lucida drawings.

Plate 4 shows the formation and growth of branched cells. The sixteen drawings were made over a period of $33\frac{1}{2}$ hours, and depict changes which occurred in the same bacterial cells throughout

that time. The cells studied were lying at the periphery of an old colony on plain meat-extract agar. They elongated slowly, and where branching was initiated the cell-tip broadened and produced small prongs which became gradually longer. The two prongs did not always develop at the same rate. Branched cells were usually strongly Gram-positive. Different branched cells differed markedly in form (figs. 1, 3, 6 and 8, Plate 1). In some instances filaments showing multiple branching were observed.

Figures 12 and 13 (Plate 2) represent a variant cell type which developed spindle-shaped swellings. The cells shown in these photographs were located at the edge of a colony growing on a hanging block. The same microscopic field is represented in both figures, but photograph 13 was made seven hours later than photograph 12. It is apparent that certain of the swollen cells were not dead, and that they were able to develop slowly during the seven-hour interval. Under certain circumstances practically all of the cells around colony peripheries were of this type. Similar forms have been described in the literature for other species. Kühn and Sternberg (1931) stated that such swollen cells developed when the spores of a protozoan parasite in the bacterial culture penetrated the bacterial cells and grew there. No evidence suggesting a protozoan parasite was found in our cultures, however.

Under certain circumstances many pointed cells developed at the peripheries of colonies. Photographs 24 and 25 (Plate 3) were selected from one of several long series of photomicrographs showing the development of such cells. Points always developed on the ends of the cells which were furthest away from the centers of the colonies. The tips of such cells seemed to push out into the medium and to become increasingly thin. Occasionally, very thin, hair-like filaments were seen. They may have represented the extreme limit of point formation.

When the ends of rods or filaments gradually swelled, clubbed organisms were produced (figs. 14 and 15, Plate 2). Clubs developed either on one or on both ends of cells.

Several types of globular cells were observed (figs. 10, 11 and 14). One type was formed from the unautolysed, swollen ends of

clubbed cells after the unswollen rod portions of the cells had autolysed and disappeared (fig. 14, Plate 2). The formation of two other globular cell types was described by Rettger and Gillespie in 1935.

Gram stains were made on material from seventy-day-old broth cultures. Variant cell types similar to those found in old agar cultures were observed. Long rods and short filaments predominated. Branched forms, clubbed cells, pointed rods, bulged filaments, and long twisted or braided cells were also seen. Since most of the organisms present were strongly Gram-positive, it may be assumed that numerous living and vigorous micro-organisms were present in the old broth cultures, in spite of the supposedly unfavorable conditions surrounding them.

All of the variant cells studied developed from apparently normal rods and filaments. Organisms of unusual shape were never seen to produce more cells of the same type, and one form of variant cell never developed into another form of variant cell. In the environment responsible for their formation such cells simply increased in size up to a certain point, and then lay dormant until death and autolysis ensued. Different morphological types seemed to form in response to slight variations in the environment in which growth was occurring.

FACTORS INCITING CELLULAR VARIATION

In 1935 Rettger and Gillespie emphasized the variation-inciting effect of some factor or group of factors associated with growth crowding. Throughout the course of this investigation the existence of a direct relationship between growth crowding and the development of cellular variants was again clearly apparent. As the number of organisms in colonies increased, the medium surrounding the colonies became progressively less favorable for growth. Atypical cells of various kinds appeared at the peripheries of colonies just before reproduction and growth ceased. No variation occurred when conditions suddenly became so unfavorable that growth was completely stopped. Under such severe conditions cell development at once ceased, and practically all of the cells in the colonies died.

The following theories may be advanced to explain the influence of growth crowding on cellular variation.

1. Crowding may result in the production of large amounts of toxic metabolites, and the accumulation of these substances may incite pleomorphism.

2. Crowding may cause the exhaustion of essential food materials, and nutrient starvation may lead to morphological variation.

3. Crowding may be accompanied by oxygen depletion, and an insufficient oxygen supply may be responsible for variant cell formation.

4. Crowding may alter the oxidation-reduction potential of the medium, and the resulting unfavorable potential may call forth variant types.

5. Crowding may hasten the maturation of the cells in the culture, and the pleomorphic phases of a reproductive cycle may appear sooner under such circumstances than they would in a less crowded environment.

6. A combination of these factors may operate.

Repeated attempts were made to demonstrate the influence of one or more of the factors mentioned above. In order to investigate the effect of non-gaseous toxic metabolites, twenty-five micro-cultures were grown on agar blocks containing filtrates from broth cultures of *B. megatherium.* Twenty-four hanging block cultures were also made with agar containing the supernatant fluid from centrifuged broth cultures, because it was feared that toxic metabolites might have been removed from the filtered culture fluids by adsorption. Broth cultures two, nine, fourteen, fifty-nine, and one-hundred-and-two days old were used. Certain of the filtrates and supernatant fluids were autoclaved before use; others were not heated beyond 45°C. The autoclaved fluids were not diluted, whereas the unheated fluids were used in varying concentrations. Agar blocks made with unheated supernatant fluids were, for the most part, not inoculated, because many living cells which were not removed by the centrifugation were still present in the medium. Cell growth in some of the cultures was followed for as long as thirty days.

Morphological variants appeared in all of the cultures at one

time or another, but the time of their appearance, their nature and their prevalence could not be related to any factor other than simple growth crowding. On the one-hundred-and-two-day-old filtrate blocks the colonies which developed were relatively small, but even after eleven days many hyalin and apparently vigorous cells could be seen. The medium seemed on the whole to bring about growth limitation, rather than cell injury, although in several of the cultures extreme morphological variation occurred. The development of numerous spores and pointed cells on all of the one-hundred-and-two-day-old filtrate blocks indicated that nutrient starvation was one of the unfavorable factors involved here.

Striking colonial variants developed in the blocks containing unheated supernatant fluids. No appreciable difference in cell morphology could be observed in the different colonial types, however. Spore formation occurred in the cells of both R and S colonies.

Filterable forms were never demonstrable, although a number of different filtrates from cultures of various ages were examined over long periods.

In order to determine the effect of gaseous metabolites, and of oxygen deprivation on cellular variation, fifteen hanging block cultures were set up. Inoculated pieces of agar were placed in the bottoms of the moist chambers before the latter were sealed. Any influence on the hanging block cultures exerted by the growth on the pieces of agar on the floors of the chambers, under the conditions of these experiments, had of necessity to extend through the air in the chambers; that is, it must have been of a gaseous nature.

The results obtained in three of the fifteen experiments strongly pointed toward a variation-inciting influence of either accumulated gaseous metabolites or partial oxygen starvation. Bulged and swollen filaments appeared in large numbers (figs. 12 and 13). The effects of nutrient starvation and of non-gaseous metabolites were eliminated in these instances, since the single colonies on the hanging blocks were very small, and there was evidently no growth crowding.

The balance of factors involved in the stimulation of variation by gaseous means was apparently very delicate, however. It was exceedingly difficult to duplicate the results obtained. In most instances no variation occurred in the cultures on the hanging blocks, because the abundance of growth on the pieces of agar resting on the bottoms of the closed chambers caused all the cell development in the chambers to stop very quickly.

Rettger and Gillespie (1935) observed the formation of certain coccoid cells in cultures of *B. megatherium* under conditions which favored gradual oxygen depletion and the accumulation of volatile metabolites. In order to distinguish between the effect of oxygen starvation and that of accumulated gaseous metabolites, microscope-stage moist chambers to which two "side-arm" glass tubes had been sealed were employed. Bottles containing alkaline pyrogallol were attached to each of the side arms. In some of the experiments the preparations were made air tight; in others air was admitted through small pin-holes in the paraffin seal. No striking cellular variants appeared; however, the cells at the colony peripheries became very short. The decrease in cell length could not, under these circumstances, have been due to the accumulation of gaseous metabolites, because the air space in the chamber was supplemented by that in the two bottles. It appeared to be due to simple oxygen starvation.

In order to study the influence of nutrient starvation on cellular variation, fifty-eight cultures were grown on agar blocks which contained from ten times to one one-thousandth of the usual concentration of nutrients (see page 41). The only cell-types which seemed to be directly related to nutrient scarcity were spores and pointed rods (Gillespie and Rettger, 1937). Within certain limits, decreased nutriment increased the proportion of these forms. Furthermore, they never appeared in appreciable numbers on media containing concentrated nutriment. Nevertheless, every cell type appeared at one time or another on practically all of the media studied. Only those cultures which were made on media containing too little food to support growth failed completely to show variant cell forms.

BEHAVIOR OF VARIANT CELLS WHEN TRANSFERRED TO NEW AND FAVORABLE ENVIRONMENTS

When transferred to a fresh and favorable medium, viable variant cells always returned rapidly to the "normal" rod form. Branched cells on a fresh nutrient block showed the "three point" mode of development described by Gardner in 1925. Figures 1 through 9 (Plate 1) were taken from several series of photomicrographs showing the development of individual branched cells. The branch itself usually remained intact for some time, while each of the three free ends elongated and produced a series of rods. Occasionally one or two of the prongs failed to grow. Segmentation eventually occurred at the point of branching, and only chains of rods remained (figs. 2 and 9). When the prongs of a forked cell tapered (figs. 1 and 2), the tapering tendency continued for some time during the growth of the "points" (fig. 2).

The growth and reproduction of a typical clubbed cell is clearly shown in figures 15 through 18 (Plate 2). The photomicrographs presented here were selected from one of several long series showing the same phenomenon. Clubbed cells usually developed rapidly when placed on fresh medium. Both the swollen and the thin end of each organism elongated. Chains of thick rods were formed from the clubbed ends, whereas the cells formed from the narrower ends were appreciably thinner. Furthermore, the thick cells invariably showed a peculiar tendency to curl or twist. How long the differences in cell breadth persist has not as yet been determined. Practically all of the clubbed cells studied formed a peculiar small knuckle-like projection on the side of the first thick organism which developed as the result of the elongation of the swollen end of the club. The projection always developed in the vicinity of the first line of division. It persisted for some time as a globular swelling on the end of one of the newly formed rods. Figures 16 and 17 illustrate this phenomenon clearly.

Figures 26 and 27 (Plate 3) show the fate of two pointed cells. In one instance the point did not develop, and was apparently cast aside. In the other the part of the cell immediately back

of the tip revealed a peculiar transverse type of swelling. Both of these modes of development were often observed in other cells of the same type.

Although many granules and granular cells were seen in various preparations, no evidence suggesting the existence of granules which were capable of reproduction was obtained.

SUMMARY AND CONCLUSIONS

The classical or so-called "normal" rod form of *Bacillus megatherium* appeared when the organism was grown in a reasonably favorable environment. It was only after growth and reproduction had practically ceased, due to some inhibiting influence, that variant cell formation and sporulation occurred.

The following variant types were seen: (1) coccoid cells, (2) globular and balloon-like bodies, (3) pointed bacilli, (4) branched organisms, (5) clubbed rods, (6) filaments with bulbous swellings, and (7) bizarre forms. The organisms in groups 3, 4 and 5 ranged in length from short rods to long filaments.

Cells of unusual shape never produced new organisms of the same type. In the environment responsible for their formation they simply increased in size up to a certain point, and then remained dormant until death and autolysis ensued. When transferred to new and wholesome surroundings, viable variant cells returned to the "normal" rod form.

When planted on fresh nutrient agar the stem and both prongs of branched forms frequently elongated and broke up into ordinary bacilli. Clubbed organisms also increased in size and segmented into rods in new, favorable environments. The cells which developed from the swollen ends of the clubs were considerably thicker than those which came from the opposite ends. Furthermore, the thick cells curled and twisted in an unusual fashion. Very few globular cells were seen to develop. Those which did grow returned, after a few divisions, to the "normal" rod form. A peculiar twisting or braiding-together of long filaments was observed. The factors responsible for this appeared to be purely mechanical.

When sufficient Bacto-agar was added to old broth cultures of

the organism to convert the medium into solid agar, extremely rough, as well as intermediate, colonies developed. The colony variants were made up of normal-appearing rods. No definite relationship seemed to exist between cellular and colonial variation.

The relation of growth crowding to the stimulation of cellular variation was re-emphasized. Hoping to determine the environmental factors which were responsible for cellular variation, attempts were made to separate the influences which might be associated with growth crowding, and to stimulate variation by applying those influences individually to growing cells.

Homologous metabolites of various ages in filtered broth cultures and in centrifuged unfiltered culture fluids were made up into solid agar media. Surprisingly luxuriant and normal growth appeared on these media. No clear-cut evidence was obtained which indicated that accumulated toxic metabolic products were responsible for cellular variation. It is possible, however, that some easily oxidized or otherwise unstable substances may have been involved.

The rôle of either accumulated gaseous metabolites or of partial oxygen deprivation as incitants to the production of bulged and swollen filaments was strongly suggested. The effect of nutrient starvation was investigated also. Pointed rods and spores appeared regularly on nutrient-poor media, but no other cell-types could be brought out by varying the amount of the food substance supplied.

Further evidence was obtained to support the authors' earlier claims that partial oxygen starvation leads to the formation of globular and coccoid cells. Attempts to produce other types of cellular variation by regulating the oxygen supply were unsuccessful.

Cells of unusual shape seemed to form in response to unfavorable environmental conditions. They were presumably simple adaptive variants, and did not appear to represent stages in an orderly life-cycle. No evidence was obtained which suggested the existence of viable granules.

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PLATE 1

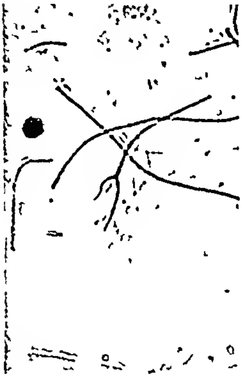
THE GROWTH AND REPRODUCTION OF BRANCHED CELLS ON HANGING BLOCKS OF PLAIN EXTRACT AGAR. ROOM TEMPERATURE. × 475.

FIGS. 1 AND 2. Cells taken from a 34-day-old agar slant culture. Period of observation, eight and one-half hours.

FIGS. 3 THROUGH 5. Cells taken from a 51-day-old agar slant culture. Period of observation, eight hours.

FIGS. 6 AND 7. Cells taken from a 28-day-old agar slant culture. Period of observation, six and three-quarters hours.

FIGS. 8 AND 9. Cells taken from a 15-day-old agar block culture. Period of observation, twenty and one-half hours. × 950.



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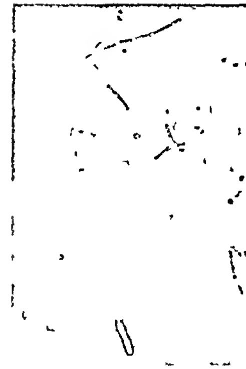

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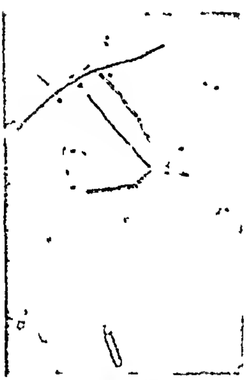

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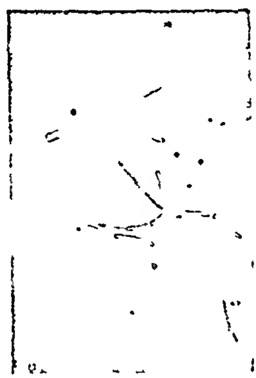

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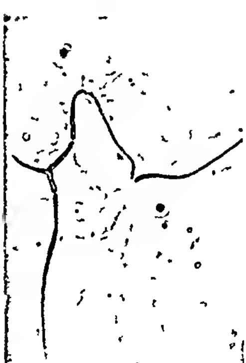

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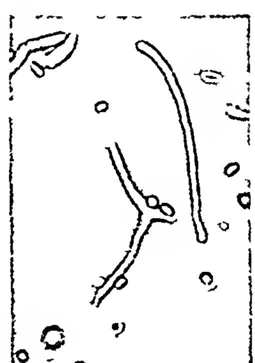

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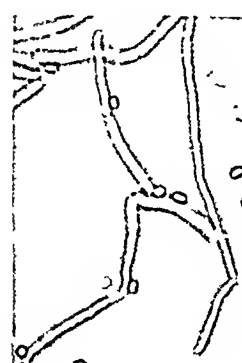

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(Hazel B Gillespie and Leo F Rettger Bacterial Variation)

PLATE 2

PHOTOMICROGRAPHS OF CELLS GROWING ON HANGING BLOCKS OF AGAR

FIGS. 10 AND 11. Swollen, oval, and globular cells in old cultures on plain extract agar blocks. Temp 30°C × 950.

FIGS. 12 AND 13. Development of cells with spindle-shaped swellings. Room temperature. × 760.

FIG. 14. Clubbed and globular cells in 15-day-old colony on plain extract agar. Temp. 30°C. × 760

FIGS. 15 THROUGH 18 Development of a clubbed cell after transfer to fresh medium. Period of observation, nine and a half hours Room temperature × 750.

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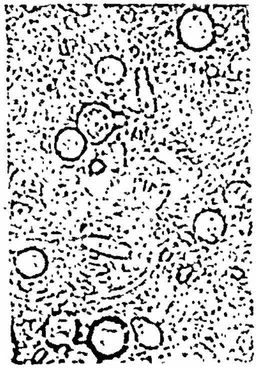

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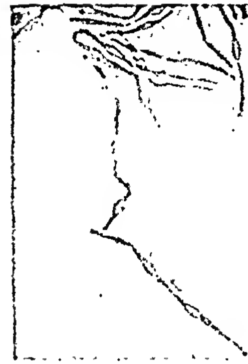

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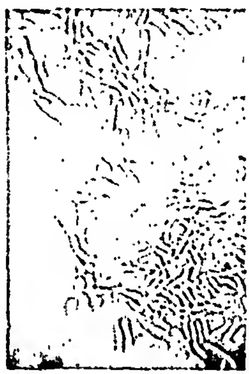

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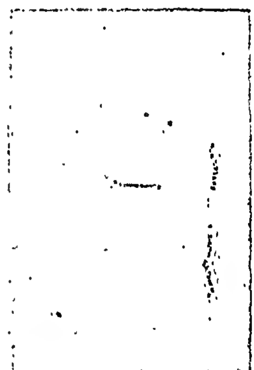

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(Hazel B. Gillespie and Leo F. Rettger: Bacterial Variation)

PLATE 3

PHOTOMICROGRAPHS OF CELLS GROWING ON HANGING BLOCKS OF AGAR

FIGS. 19 THROUGH 23. Formation of twisted filaments on Savita agar. Period of observation, three hours. Room temperature. × 760.

FIGS. 24 AND 25. Formation of pointed cells on agar containing only 0.01 of the usual amount of nutriments. Period of observation, thirty-five hours. Temperature 30°C. × 950.

FIGS. 26 AND 27. Development of pointed and globular cells. Room temperature. × 950.

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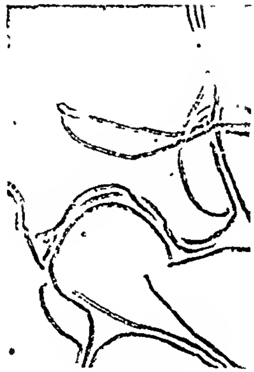

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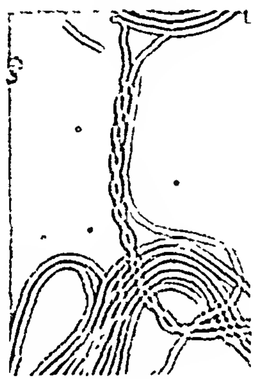

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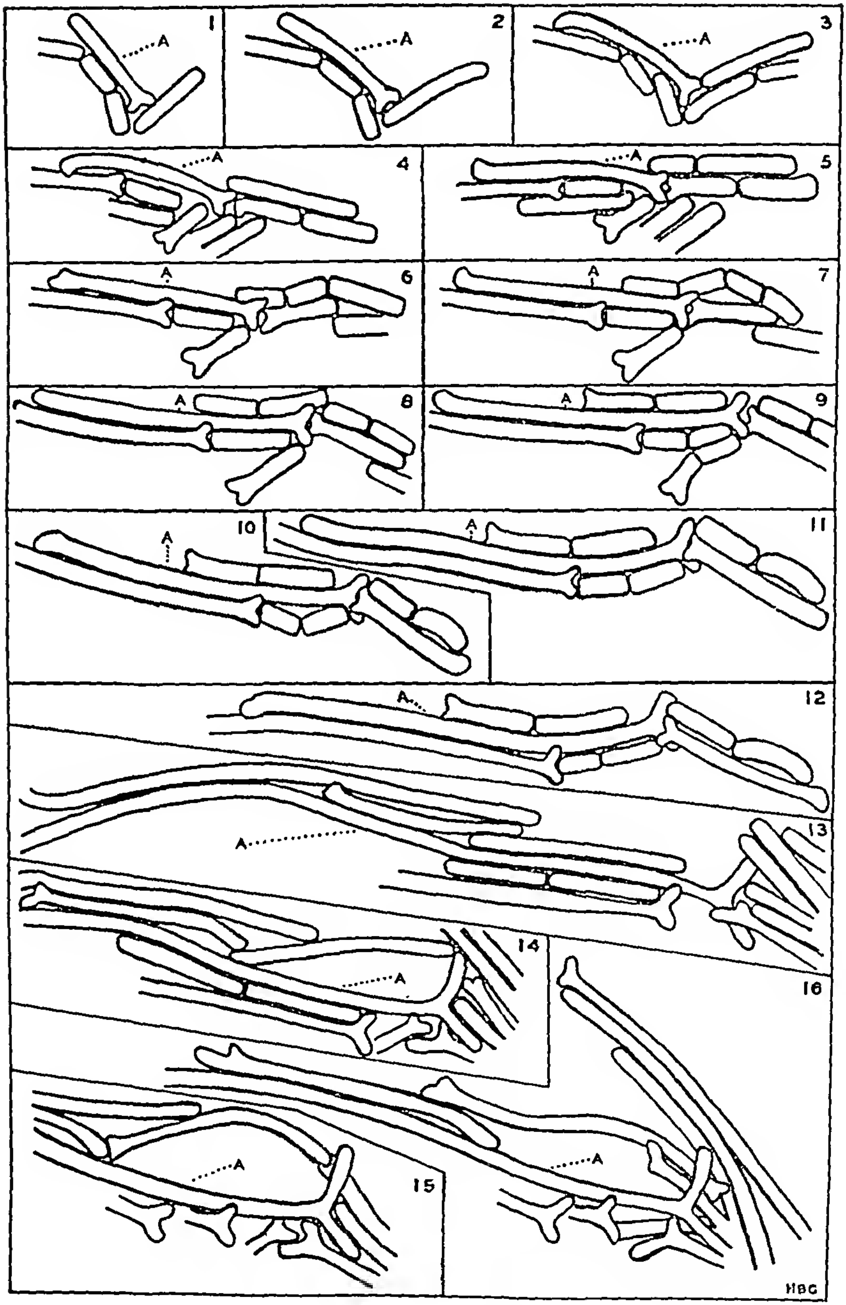
(Hazel B. Gillespie and Leo F. Rettger: Bacterial Variation)

PLATE 4

Camera lucida drawings showing formation and growth of branched cells at the periphery of an old colony on plain extract agar Period of observation, thirty-three and one-half hours Room temperature × 1500



(Hazel B. Gillespie and Leo F. Rettger: Bacterial Variation)

(1939) who used it successfully for the isolation of specific phases from all of the so-called totally and permanently nonspecific varieties of *Salmonella*. Since the Wassén technique is so greatly superior to other methods for the isolation of phases which are suppressed under ordinary methods of culture, the writers have used it to investigate phase variation in the supposedly monophasic specific types. *Salmonella abortus-equi* was chosen as the first species to be examined. The reason for this choice is obvious. The flocculating antigens of *S. abortus-equi* (*enx* of the Kauffmann-White schema) are those present in the beta phase of almost all the types which possess alpha-beta phase variation. It would thus appear that *S. abortus-equi* is a type in which the alpha phase is suppressed. This hypothesis was advanced by Kauffmann (1938), who examined several cultures of this species but could demonstrate no variation.

MATERIALS AND METHODS

From the collection of Professor E. S. Good, who very generously supplied cultures, and from specimens isolated in this laboratory, 24 strains of *S. abortus-equi* were available for study. Of these 24 cultures, 6 were non-motile or only very feebly motile and 3 were rough. The remaining 15 strains of smooth, motile bacilli were typical representatives of the species. Phase variation was noted in 6 cultures, whose designations and histories are as follows: M1, From aborted fetus, isolated 1924. M5, Isolated from aborted fetus by Dr. K. F. Meyer in 1916. McC, From aborted fetus, isolated 1923. WH2, From aborted fetus, isolated 1931. 4K88, Received from Army Medical School. Isolated from aborted fetus in 1933. P, From aborted fetus, isolated 1913.

These cultures were inoculated into semi-solid agar containing various antiserums by stabbing along one side of the tube. In the original experiments, *S. abortus-equi* antiserum which had been freed of somatic agglutinins by absorption with the Reading type was added to the agar. In later trials serum derived from the beta phase of the Minnesota type was substituted. The antigenic formula of this type was established by Edwards and

Bruner (1938) as XXI: $b \rightleftharpoons enx$. Thus the flocculating antigens of the beta phase of Minnesota are similar to those naturally occurring in *S. abortus-equi*. These serums were used in amount sufficient to confine the growth of the normal phase of the bacilli to the line of inoculation. Outgrowths from the line of stab represented the appearance of hitherto masked phases. As these phases appeared they were purified by continued transfer and agglutinating serums were prepared for them. These serums, after absorption with appropriate bacilli, and *Salmonella paratyphi A* serum were in turn added to semi-solid agar for use in attempts to revert the induced phases.

RESULTS

Before the results of the experiments can be given, some designation must be assigned the phases isolated. Kauffmann (1938a) has revised the Kauffmann-White schema so that the phases of diphasic organisms are referred to as phase 1 or phase 2. In the case of the types which exhibit specific-nonspecific variation the specific antigens are designated as phase 1, the nonspecific as phase 2. In organisms which display alpha-beta variation, the alpha components are called phase 1, the beta are denoted as phase 2. Since the normal antigens of *S. abortus-equi* (*enx*) are those found in phase 2 of the revised schema, they will be designated as phase 2. Two other phases which were found will be designated as phase 1 and phase 3. As will be shown later, phase 1 is identical with the normal flocculating antigen of *S. paratyphi A* (*a*), while phase 3 is closely related to the antigen which has been known as the beta phase of the Schleissheim type (*z*5 of Kauffmann and Tesdal, 1937).

Phase 1 was recovered from each of the six cultures listed above. Phase 3 was recovered from four cultures; M5, McC, WH2, and P. No special effort was made to recover phase 3 from cultures M1 and 4K88. Not only was it possible to recover these hitherto suppressed antigens from the cultures, but by using a suitable serum or combination of serums the phases could be transformed from one into another at will. The degree of control which it was possible to exercise over phase variation in these

cultures was surprising. It is unnecessary to give in detail the results obtained with all the cultures. The results obtained with one culture are presented in figure 1.

From figure 1 it can be seen that either phase 1 or phase 3 can be obtained from the naturally-occurring phase 2 and that both of these artificially induced phases may be reverted to the original phase or transformed to the other induced phase as desired.

$$
\begin{array}{c} \textit{S. abortus equi}\ \text{M5} \\ \text{Phase 2} \end{array}
\begin{cases}
\xrightarrow{enx\ +\ a} \text{Phase 3} \begin{cases} \xrightarrow{a\ +\ z5} \text{Phase 2} \\ \xrightarrow{enx\ +\ z5} \text{Phase 1} \end{cases} \\
\xrightarrow{enx\ +\ z5} \text{Phase 1} \begin{cases} \xrightarrow{a\ +\ enx} \text{Phase 3} \\ \xrightarrow{a\ +\ z5} \text{Phase 2} \end{cases}
\end{cases}
$$

FIG. 1. PHASE VARIATION INDUCED IN S. ABORTUS EQUI

The symbols on the arrows indicate the serums added to the medium.

enx—antiserum derived of the beta phase of the Minnesota type or antiserum derived from phase 2 of *S. abortus equi*. The latter serum was absorbed by the Reading type before use.

z5—antiserum derived from phase 3 of *S. abortus equi*. This serum was absorbed with phase 2 of *S. abortus equi* before use.

a—antiserum derived from *S. paratyphi* A.

$$
\begin{tikzcd}
 & \text{Phase 1 } (a) \arrow[dl, shift left] \arrow[dr, shift left] & \\
\text{Phase 2 } (enx) \arrow[ur, shift left] \arrow[rr, shift left] & & \text{Phase 3 } (z5\ \ldots) \arrow[ul, shift left] \arrow[ll, shift left]
\end{tikzcd}
$$

FIG. 2. THE REVERSIBILITY OF THE PHASES OF S. ABORTUS EQUI

The symbols accompanying the phases indicate the antigens which they contain. These are designated in accordance with the Kauffmann-White schema.

Unless the proper combination of serums is used a mixture of phases may result. For instance, if phase 1 is grown in *S. paratyphi A* serum alone, both phase 2 and phase 3 may be obtained from it. The phases of *S. abortus-equi* and the changes which have been induced in them are indicated in figure 2.

The serological relationships of the phases were examined by agglutination and agglutinin absorption. It should be emphasized that the changes described in the organisms apply only

to the heat-labile or flocculating antigens. The heat-stable or somatic antigens remained unchanged throughout the course of the work. Agglutination and agglutinin absorption tests proved that the somatic antigens of phases 1, 2 and 3 were identical.

TABLE 1

Agglutination tests

ANTIGENS	ANTISERUMS					
	Abortus equi M1, phase 1	*Abortus equi* M5, phase 2	*Abortus equi* M5, phase 3	*Paratyphi A*	Minnesota-beta	Schleissheim-beta
Abortus equi M5—phase 1...	20,000	500	200	20,000	0	0
Abortus equi M5—phase 2...	0	10,000	500	0	20,000	0
Abortus equi M5—phase 3...	0	1,000	5,000	0	0	2,000
Paratyphi A...............	20,000	0	0	20,000	0	0
Minnesota-beta..............	0	10,000	0	0	20,000	0
Schleissheim-beta...........	0	0	2,000	0	0	5,000

0 indicates no agglutination at 1:200.

TABLE 2

Agglutinin absorption tests

ANTIGENS	ANTISERUMS											
	Abortus equi M1, phase 1, absorbed by			*Abortus equi* M5, phase 2, absorbed by			*Abortus equi* M5, phase 3, absorbed by				*Paratyphi A* absorbed by *Abortus equi* M5, phase 1	Schleissheim-beta absorbed by *Abortus equi* M5, phase 3
	Abortus equi M5, phase 2	*Abortus equi* M5, phase 3	*Paratyphi A*	*Abortus equi* M5, phase 1	*Abortus equi* M5, phase 3	Minnesota-beta	*Abortus equi* M5, phase 1	*Abortus equi* M5, phase 2	Schleissheim-beta	Schleissheim-beta and *Abortus equi* M5, phase 2		
Abortus equi M5—phase 1...	10,000	10,000	0	0	0		0	0	0			
Abortus equi M5—phase 2..	0	0		10,000	10,000	0	200	0	500	0	0	
Abortus equi M5—phase 3...	0	0		200	0		5,000	5,000	2,000	1,000		0
Paratyphi A..............	10,000	10,000	0								0	
Minnesota-beta............				10,000	10,000	0						
Schleissheim-beta..........							2,000	2,000	0	0		2,000

0 indicates no agglutination at 1:200.

The relationships of the flocculating antigens are given in tables 1 and 2. Except for the relationships shown in table 1, the 3 phases of *S. abortus-equi* failed to react in significant degree with any of the antigens of the Kauffmann-White classification.

Naturally, they reacted with all types containing the antigens *a*, *e*, *n* or *x*. Although the three phases show more or less relationship to each other they are easily distinguished. While serum derived from phase 1 fails to agglutinate phase 2 or phase 3, serums derived from both phases 2 and 3 agglutinate phase 1 in low dilution. The closest relationship is between phase 2 and phase 3. The agglutination and absorption tests demonstrate the identity of phase 1 with the flocculating antigen of *S. paratyphi A*. Phase 2, the only phase of the bacillus which is known to occur naturally, is identical with the flocculating antigens of the beta phase of the Minnesota type. It is surprising that while the flocculating antigens of phase 2 and of Minnesota beta are identical, phase 3 is agglutinated by phase 2 serum but not by Minnesota beta serum. Phase 3 is closely related to the beta phase of the Schleissheim type but, as demonstrated by absorption tests, the two are not identical. The phases, when once isolated, are quite stable. It is obvious that phase 2, which occurs naturally, is quite stable since it is the only form in which the organism has been found previously. Phase 1 and phase 3 are apparently equally constant. No variation has been observed in these phases except when they were cultivated in semisolid agar containing their respective antiserums. This stability of the phases under ordinary conditions of culture supports the view that changes observed when they were placed in contact with appropriate serums were actually due to reversion and not to impurity of the phases. The behavior of the phases in semisolid agar containing immune serum also supports the view that reversion actually occurred. A species which is naturally diphasic spreads readily through semisolid agar containing serum which acts upon the predominant phase. Even the Binns type, whose stubborn non-specificity is notorious, migrates rapidly through a medium containing serum which immobilizes the non-specific phase. The phases of *S. abortus-equi*, on the contrary, act in an entirely different manner. The initial cultures show only one or two small filmy protrusions from the line of stab, and these usually appear after the tubes have been incubated for 48 hours or more. Such behavior strongly indicates actual reversion.

DISCUSSION

It is difficult to discuss the facts presented above because the mechanism upon which phase variation rests is not understood. Nevertheless several points call for comment. First, there is the demonstration of more than two phases in a single bacterial culture. So far as the writers are aware, no more than two phases have been reported previously. The demonstration of a third phase suggests that eventually a still larger number may be found. The significance of this observation is not clear. It may be said to add support to the view of White (1926) that the paratyphoid bacilli as we know them today are loss variants of primitive types which had wide antigenic relationships and little or no host specificity. White further postulated that, as these primitive types became adapted to specific hosts, certain antigens were lost and that the types most specialized in their host relationships were likely to exhibit restricted antigenic components. The monophasic typhoid bacillus, *S. paratyphi A* and *S. abortus-equi* were cited as examples. If this theory of *Salmonella* phylogeny is accepted it must be realized that the phases which have disappeared are not actually lost, but are merely suppressed. In further support of this view it may be mentioned that the writers have isolated nonspecific phases from *S. paratyphi A*, which has hitherto been considered monophasic. The work on this species is not sufficiently advanced to warrant a detailed report.

Kauffmann (1936) demonstrated a beta phase in one culture of the typhoid bacillus by cultivation in broth containing immune serum for the Muenchen type. Efforts to isolate the beta phase from other cultures resulted in failure. Further, the induced phase could not be reverted to the normal alpha phase. Attempts to induce phase variation in *S. paratyphi A* were unsuccessful. In commenting on these results Kauffmann expressed the view that *S. paratyphi A*, like the typhoid bacillus, was capable of phase variation and that possibly all *Salmonella* types were diphasic or multiphasic. He also pointed out the possibility that diphasic strains such as *S. paratyphi B* or *S. paratyphi C* could be induced to yield beta variants of the specific

antigens by cultivation in suitable immune serums. The present work is partial proof of that hypothesis.

Kauffmann (1938a) has also advanced the opinion that the flocculating components of any *Salmonella* type are complex and consist of a number of partial antigens, one or two of which predominate and determine the character of the phases. He believes that it is possible by cultivation in certain immune serums to subordinate the dominant antigens and promote those which ordinarily are of minor importance. Thus, it would be possible to obtain a number of phases from a given type, corresponding to the partial antigens of that type.

While immune serums were used to isolate beta phases from the typhoid bacillus by Kauffmann (1936), from the Schleissheim type by Kauffmann and Tesdal (1937) and from *Salmonella abortus-canis* by Gard (1938), the work reported here constitutes the first instance in which phases obtained from apparently monophasic specific types have been demonstrated to be reversible. The reversibility of the induced antigens indicates that the phenomenon being dealt with is a true example of phase variation and not merely a mutation or degeneration produced through cultivation in immune serum. The fact that phase 1 is identical with the normal flocculating antigens of *S. paratyphi A*, and that this component frequently occurs in diphasic cultures whose beta phase is identical with phase 2 of *S. abortus-equi* is a further indication that a true phase variation has been discovered.

The relationship of phase 3 of *S. abortus-equi* and the beta phase of the Schleissheim type holds added interest when it is considered that both are induced phases. Likewise the beta phase of *S. abortus-canis* of Gard (1938) and the beta phase of the Schleissheim type are induced phases which are more or less related to each other. Similarly the writers have been able to isolate a beta phase from *S. paratyphi A* and an induced phase from the Kentucky type which are closely related. After more work has been done on this problem it is possible that phases isolated with the aid of immune serums will be found to follow a recurring pattern, as do the naturally occuring antigens of the Kauffmann-White schema.

The discussion would not be complete without some reference to the relation of this work to transformation of types within the genus. The demonstration of phase 1 in *S. abortus-equi* endows this species with all the major antigenic components of the naturally diphasic Bispebjerg type, whose antigenic formula is IV: $a \rightleftharpoons enx$. All efforts to demonstrate a third phase in the one available culture of the latter type have failed. The two types differ in their biochemical activity and while closely related serologically, they are not identical. Although it is entirely probable that further unsuspected antigenic relationships between different types will be established by work similar to that reported here, it seems improbable that actual transformation of types will be accomplished in this manner.

CONCLUSIONS

Study of the supposedly monophasic *Salmonella abortus-equi* by the Wassén technique revealed that this organism contained three phases. Phase 1 is identical with the flocculating antigens of *Salmonella paratyphi A*. Phase 2 is the form in which the species naturally occurs. Phase 3 is related to, but not identical with, the beta phase of the Schleissheim type. The phases are reversible and by use of suitable immune serums, one phase may be changed to either of the others. This is the first instance in which more than two phases have been demonstrated in a *Salmonella*. It is also the first instance in which induced phases isolated from monophasic specific types have been proved to be reversible.

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STUDIES ON THE PROACTINOMYCES[1]

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The taxonomy of the actinomycetes has been subject to unending controversy, and further confusion has been introduced by the formation of a group intermediate between *Actinomyces* and *Corynebacterium* and *Mycobacterium* which was termed *Proactinomyces*. It is to the validity and composition of this group of organisms that these studies were directed. Two virtually identical systems of separation, differing only in nomenclature and using morphological characters for their main subdivisions, are those proposed by Orskov (1923, 1938) and Jensen (1931a and b, 1932) illustrated in table 1. Since the nomenclature of Jensen seems based on sounder taxonomic principles it has been used in this paper. The earlier physiological studies of Waksman (1919) and the morphological studies of Drechsler (1919) were confined essentially to the "Actinomyces" group of Jensen and may serve, therefore, as further physiological subdivisions of this group, with the exception of one culture, "*Act. asteroides*," which is a *Proactinomyces*.

The members of the genus *Proactinomyces* are distinguished from the members of the genus *Actinomyces* by the former's inability to form spores in the aerial mycelium. Spores are defined as "bodies identical to one another in form having a special mode of formation" and are to be distinguished from the so-called fragmentation "spores." Eighteen strains of acid-fast and 11 strains of non acid-fast *Proactinomyces* which sometimes

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formed aerial mycelia, together with 10 species selected at random from among the true *Actinomyces*, were subjected to a wide variety of cultural and environmental conditions to determine whether some condition might not be found in which spore formation could be induced. These were cultivated, at 28°C., in tubes, plates and slide cultures, upon *Czapek's*, *starch*, egg *albumin*, nutrient, glycerol and water agars, as described by Waksman

TABLE 1

Nomenclature of actinomycetes

<table>
<tr><th>CHARACTER</th><th>ORSKOV (1923, 1938)</th><th>JENSEN (1931a and b, 1932)</th></tr>
<tr><td>I. Spore formation in aerial mycelium</td><td rowspan="2">Micromonospora</td><td rowspan="2">Micromonospora</td></tr>
<tr><td>A. Single spores born at end of branch</td></tr>
<tr><td>B. Spores formed in chains at end of mycelial hyphae</td><td rowspan="2">Cohnistreptothrix</td><td rowspan="3">Actinomyces</td></tr>
<tr><td>a. Vegetative mycelium undivided</td></tr>
<tr><td>b. Vegetative mycelium divided</td><td rowspan="10">Actinomyces</td></tr>
<tr><td>II. No spores formed in aerial mycelium</td><td rowspan="9">Proactinomyces</td></tr>
<tr><td>A. "Stable" mycelium (β form)</td></tr>
<tr><td>1. Long hyphae</td></tr>
<tr><td>2. Colony growth in liquid media</td></tr>
<tr><td>3. Actinomyces-like colony</td></tr>
<tr><td>B. "Unstable" mycelium (α form)</td></tr>
<tr><td>1. Short mycelium if formed at all</td></tr>
<tr><td>2. Bacterial (diffuse) growth in liquid media</td></tr>
<tr><td>3. Bacteria-like colony</td></tr>
</table>

(1919) as well as on potato slants and on Frazier's gelatin (Fred and Waksman, 1928). They were observed for a period of three months at approximately weekly intervals for spore formation, both by direct microscopic observation and by use of the Orskov contact cover-slip technic (1923). In no case was there any indication of spore formation among the *Proactinomyces* strains. Under similar conditions, strains of true *Actinomyces* produced

a macroscopic (in a few cases only microscopic) aerial mycelium which showed abundant spore formation within a week. In a few cases, especially on rich media, spore formation was slightly delayed, but was generally evident by the second week. Therefore, pending valid evidence to the contrary, the line of distinction between the *Proactinomyces* and the *Actinomyces* proper is probably a sufficiently constant and practical distinction to be of value in their separation.

The genus *Proactinomyces* is separated from *Corynebacterium* and *Mycobacterium* upon the basis of the absence of mycelial formation in the latter. While, admittedly, such a distinction appears eminently suitable, it has in practice resulted in considerable confusion, which arises, in part, at least, from the lack of recognition that many bacteria, when placed under conditions which somewhat restrict free movement, may (especially in early stages of growth), form structures which may be easily mistaken for mycelium. The observations of McCarter and Hastings (1935), of Krassilnikow (1934), and even of Orskov (1938) on his group "IIb" leave little doubt that strains which would generally be considered bacteria, may form, under given conditions of growth and given techniques of observation, structures which have been called "mycelium." The lack of an adequate definition of what constitutes a mycelium is a real cause of confusion. A "chain" or alignment of cells extending no more than 5–10 cell lengths and rapidly disintegrating so that the structures are not detectable after 18 to 26 hours is often considered equivalent to a mycelium 200 to 300 μ long which remains without marked fragmentation for several weeks. Jensen attempts to circumvent this difficulty by defining as *Proactinomyces* those forms which show a "constant" formation of mycelia while placing the forms showing only occasional mycelia among the bacteria. A moment's reflection will indicate that such a character is too dependent upon given cultural conditions to be of much aid in separation.

It has become increasingly apparent that the *Proactinomyces* are composed of two rather distinct types of forms, one of which is difficult to distinguish from the *Corynebacterium* or *Mycobacterium*, and which is usually termed the "unstable mycelium group"

(here called "α" *Proactinomyces*). The other group can be distinguished from *Actinomyces* by lack of spore formation, but its properties do not greatly resemble those of the first type. This latter group is usually termed "stable mycelium group" (here called "β" *Proactinomyces*). A list of the properties of these two groups is given in table 1.

Since it is recognized that simple mycelium formation is not an adequate criterion for separation from the *Corynebacterium* and *Mycobacterium*, and since the α and β groups of *Proactinomyces* are distinguished by the rather vague distinctions of "stable" and "unstable" mycelium or "slow" and "rapid" disintegration, a search for better criteria seemed desirable. Of these, three appeared suitable: (1) aerial mycelium, (2) diffuse growth in liquid media, (3) structure of colony. Aerial mycelium was present quite consistently (though sometimes microscopic) among the "stable mycelium" group, while it was never found among the "unstable mycelium" types. This character, if sufficiently constant, could be a desirable characteristic for separation. While such is Orskov's contention (1923, 1938), strains are found which show typical β structure (and virtually no signs of disintegration even when cultured for as long as 3 to 4 months) yet never produce even traces of microscopically observable aerial mycelium. Other strains show aerial mycelium only under particular *circumstances, so that production of aerial mycelium* cannot actually be used as a criterion for separation, since it is dependent, to a large extent, on the cultural conditions.

The lack of diffuse growth in fluid media seems a more suitable index of mycelium formation and it is surprising that it has not been previously used as a method of separation. It has been frequently noted that while bacteria always produce some degree of turbidity, actinomycetes do not. These show rather flaky or colony-like growths, but no turbidity. In this study it was found that all β type strains produced no turbidity while all α types did.[3] Such a separation, based upon turbidity is hardly

[3] The difference between α and β types in this respect is actually more fundamental than is at first apparent. An *Actinomyces* in liquid media forms "colonies" composed in the most part of a single individual (perhaps even a single

adequate, however, since many mycobacteria produce no turbidity, since R and S cultures often differ in turbidity, and since the O/R potential of the media often determines whether a "non-turbid" pellicle or diffuse growth is observed. Yet those familiar with the growths of an actinomycete in liquid media will recognize that the complete absence of turbidity plus the formation of colony-like structures are a constant character of *Actinomyces* and a similar type of growth among the β forms of *Proactinomyces* might serve as one of the criteria of separation. That this criterion is not entirely a plus or minus character is apparent since variations have been reported (Jensen, 1931a; Topping, 1937; Wright, 1937) to occur which resulted in turbid-forming variants.

The variants of *Proactinomyces polychromogenus* (Jensen, 1931a) obtained from the Lister Institute Collection ("filamentous," "normal" and "bacterial" forms) do not show turbidity in fluid media, but are rather of the β type (stable mycelium). The cultures of Topping (1937) (no. 114R and S) belong to the α group, while the remaining report (Wright, 1937) emphasizes that the turbidity-forming variants seem distinctly unusual and are very possibly entirely comparable to the variations which one occasionally finds in bacterial cultures. Such conversions from one species to another are not too uncommon and while they serve to indicate relationships, they do not invalidate entirely the aid in separation obtained by observations on liquid media.

Finally, the difference in the consistency of the colony is quite distinct between the α and β groups, that of the α type being generally soft, translucent and gummy, whereas the β type is hard, waxy and cannot be normally separated into fragments with the inoculating needle, yet this distinction seems somewhat too vague for adequate separation.

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cell). With bacteria no colonies are formed, or if formed (as in the waxy *Mycobacteria*) may be broken down easily by shaking, since they are composed of many individuals. It is therefore possible in many cases to place an organism nearer to the genus *Actinomyces* if it shows an actinomyces-like growth in fluid media and nearer to the bacteria if the growth is bacterial. As far as our observations go, this differential character appears to be a constant one. But undoubtedly, border-line cases exist in which the investigator must resort to other characteristics.

After studying and comparing various reputed *Proactinomyces* types sent to the laboratory through the courtesy of many workers, the following diagram of their relationships may be suggested. This is very similar to that of Jensen and to that of Orskov, the fundamental points being entirely in agreement.

Corynebacterium ↓ — Unstable mycelium observable in slide culture. Turbidity in liquid media.

Mycobacterium ↓ — Line of demarkation not exactly established.

α *Proactinomyces* (non acid-fast) ↓ β *Proactinomyces* (non acid-fast)

α *Proactinomyces* (partially acid-fast) ↓ β *Proactinomyces* (partially acid-fast)

Stable mycelium "Actinomyces" growth in liquid media.

Spore formation

↓

Actinomyces

Attention is centered in this report on the β types, particularly those producing an orange to orange-red pigment, whose function in nature (aside from the pathogenic "*Act. asteroides*") can only be indicated as a probable attack on resistant compounds (Gray and Thornton, 1928; Jensen, 1932).

TYPE I. PARTIALLY ACID-FAST β PROACTINOMYCES

Jensen (1931b and 1932) found that certain forms among the *Proactinomyces* exhibited both a relatively and a partially acid-fast character; this was also encountered among certain forms in this study. It can be interpreted to mean that not only does the acid-fast character depend to a considerable extent upon the medium (in milk they are likely to be acid-fast, while on certain

synthetic media they may be non acid-fast), but also that a part of the mycelium may be acid-fast, whereas closely adjacent parts may be non acid-fast. It has been particularly noted that the aerial mycelium seems less readily decolorized than the submerged mycelium. It is decolorized in all cases, however, more rapidly by acid alcohol than is the case with the tubercle organism. A standard method of decolorizing, which has proved satisfactory for distinguishing between partially acid-fast and non acid-fast forms, consists in placing the stained slide (Carbol-Fuchsin, 2 per cent, steam for 5 minutes) in a stain jar containing 3 per cent HCl in alcohol for exactly 15 seconds. Wash in water immediately, and place in stain jar containing 0.1 per cent aqueous solution of Methylene blue for three minutes. By this method heavy clumps may not be entirely decolorized, but they offer no difficulty if the areas free from them are examined.

The organisms included in this group, in addition to being partially acid-fast, have several other related characters. They do not hydrolyze starch, which indeed seems correlated with the partial acid-fast character (Gordon and Hagan, 1936). They do not hydrolyze gelatin, and without exception possess the ability to utilize paraffin as the sole carbon source.[4] This type was represented in this study by seventeen strains as follows:

3308A. *A. asteroides.* Waksman collection.
3013. Unidentified pathogen obtained from Dr. Broadhurst.
3306. *A. asteroides* Bering.
401.[5] *A. farcinica* (Fitch 1932).
558. *A. gypsoides* (Henrici 1934).
559. *A. asteroides* (Henrici 1934).
568, 568a, 568b. Unidentified forms, isolated from potato scab in 1932 by Dr. Taylor at Cornell University.
616, 619, 631, 632, 642, 648, 649, 650. Unidentified strains isolated from soil by Dr. Gordon, at Cornell University in 1935.

[4] Paraffin utilization was determined by inoculation into a liquid medium composed of the salts employed in Czapek's media with a drop of melted commercial paraffin per tube. While this paraffin probably was not entirely pure it served to distinguish the acid-fast cultures from the non-acid-fast cultures, since the latter showed no growth whatsoever.

[5] Cultures 401 through 650 were obtained through the courtesy of Dr. W. A. Hagan, Cornell University.

The consistency of the growth of these organisms is crumbly and wax-like, as compared to the smooth, shiny, hard growths of the non acid-fast types. On Czapek's agar (sucrose-$NaNO_3$ medium), growth is scanty in the case of all strains, but sooner or later (usually within 5 days) a white aerial mycelium begins to form. Some strains produce more aerial mycelium than others but in no case was spore formation observed. The pigment of the cultures was orange to orange-red but no black color was produced on any of the media employed. There was considerable fragmentation of the mycelium after one week, at 28°C., into rod-shaped branching forms of varied lengths, but long strands were visible for as long as two months. Previous descriptions of *A. asteroides* leave little to add (Henrici, 1930; Henrici and Gardner, 1921; Waksman, 1919).

A few of the properties of the 17 strains falling into this group are summarized in table 2. Among these strains, several were described as distinct species and several were unidentified forms. Data are presented only of the growth on Czapek's synthetic agar (on which the organisms grow slowly) and on beef peptone agar (on which they grow rapidly). Growth on water-agar, paraffin agar and egg-albumin agar is almost identical to that on Czapek's; starch-ammonium sulfate and glycerol-nitrate agars are somewhat better nutrients and occupy an intermediate position, while Frazier gelatin agar gives growth very similar to that on beef-peptone. Data are given for the characters of the organisms after 5 and 20 days incubation. It will be noted that differences between the several strains are relatively minor and and in reality reflect slightly different rates of development, rather than any differences in the kind of growth; there is probably no justification at the present time, for their further separation into separate species upon the basis of growth characters.

It is entirely possible that these strains differed from the type strain *Proactinomyces asteroides* (Eppinger) Gasperini when they were first isolated, but that cultivation on artificial media has caused the loss of these differentiating characters, which indeed has been shown to be the case with "*Act. gypsoides*" (Henrici, 1930). At present, however, they may be considered as but

TABLE 2

Cultural characters of partially acid-fast β proactinomyces

NUMBER	DESCRIPTION	SYNTHETIC AGAR§										NUTRIENT AGAR§										STARCH		GELATIN		MILK‡		PARAFFIN GROWTH	
		Growth		Color*		Aerial mycelium		Acid-fast		Fragmentation of mycelium		Amount of growth		Color		Aerial mycelium		Acid-fast		Fragmentation of mycelium		Hydrolysis				Reaction	Acid-fast		
		Days of growth																											
		5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	30	30	5	20
3308A	*A. asteroides*	1	2	–	O-R	±	+	+	+	+	++	3	4	O	O	++	++	+	+	+	++	–	–	–	–	Ac	+	–	++
3013	No. 2 Henrici	±	2	O	O-Y	–	–	±	+	±	++	1	2	O	O	–	–	+	±	–	–	–	–	–	–	–	+	–	++
3306	*A. asteroides* Bering	1	2	–O	O-R	–	++	+	+	+	++	1	2	–O	O†	–	–	+	+	+	++	–	–	–	–	–	+	–	++
401	*A. farcinica*	1	2	O	O-R	+	+	±	+	±	++	3	4	O-Y	O	–	–	–	+	+	++	–	–	–	–	–	+	–	++
558	*A. gypsoides*	1	2	O	O	++	++	±	+	+	++	3	4	O-Y†	O-Y†	–	–	–	±	++	++	–	–	–	–	Ac	+	–	++
559	*A. asteroides*	1	2	O	O	±	±	±	+	++	++	3	4	O-Y	O-W†	–	–	+	+	+	++	–	–	–	–	–	+	–	++
568	Unidentified	±	2	O	O-R	+	+	±	+	+	++	3	4	O-Y	O-R	–	–	–	–	++	++	–	–	–	–	AIK	+	–	++
568A	Unidentified	1	2	O	O-R	±	±	±	+	+	+	3	4	O-Y	O-R	–	–	+	+	++	++	–	–	–	–	AIK	+	–	++
568B	Unidentified	1	2	O	O-W	±	±	–	+	+	+	3	4	O-Y	W	–	–	±	±	++	++	–	–	–	–	AIK	+	–	++
616	Unidentified	1	2	O	O-R	±	±	–	+	+	++	3	4	Y	O	±	+	–	+	++	++	–	–	–	–	AIK	+	–	++
619	Unidentified	1	2	O	O-W	++	++	±	+	++	++	3	4	O-W	O-W	++	++	±	+	+	+	–	–	–	–	AIK	+	–	++
631	Unidentified	1	2	O	O	±	±	–	+	++	++	3	4	O-W	O-W	–	–	–	+	+	+	–	–	–	–	Ac	+	–	++
633	Unidentified	1	2	O	O	±	+++	±	+	++	++	3	4	O-Y	O-W*	++	++	–	+	+	+	–	–	–	–	AIK	+	–	++
642	Unidentified	1	2	O	O	–	++	–	+	+	++	3	1	O-Y	W	++	++	+	+	++	++	–	–	–	–	AIK	+	–	++
648	Unidentified	1	2	O	O	±	++	±	+	+	+++	3	4	O-Y	O-W	–	–	±	+	+	++	–	–	–	–	AIK	+	–	++
649	Unidentified	1	2	O	O	++	++	±	+	+	+	3	4	O-Y	O-W	–	–	+	+	++	++	–	–	–	–	AIK	+	–	++
650	Unidentified	1	2	O	O	±	±	±	+	+	++	3	4	O-Y	O-W	–	–	±	+	+	++	–	–	–	–	AIK	+	–	++
3322B	Non-acid fast YPC (see table 3)	3	4	O	O	–	–	–	–	–	±	4	4	O-R	O-R	–	–	–	–	–	±	+	+	+	+	–	–	–	–

* O, orange; W, white; R, red; Y, yellow.

† Slight production of a soluble yellow pigment diffusing into media.

‡ Observations at 30 days. Slow clearing after 4 weeks. Milk remains fluid.

§ Waksman (1919).

minor variants of the well-known type *Proactinomyces asteroides*, and without doubt, should be so designated.

Jensen (1932) has proposed the delineation of four other species within this group of organisms which are defined as follows:

Initial mycelium well-developed:
1. Vegetative mycelium, soft, little aerial mycelium:
    a. Red colored (yellow or white variants), *P. polychromogenus*,
    b. White to pale pink colored *P. opacus*, *P. erythropolis*,
2. Vegetative mycelium, hard:
    a. Yellow colored *P. paraffinae*.

The descriptions given by Jensen would lead one to consider *P. polychromogenus*, *P. opacus*, *P. erythropolis* as members of the α type. Examination of "*A. erythropolis*" (American Type Culture Collection no. 4277) confirms this suspicion. However, the cultures of *P. polychromogenus* from the Lister Institute and another kindly supplied by Dr. Jensen were found to be of the β mycelium type, and closely resembled the types studied here. All of which illustrates the confusion inherent in descriptions which employ "stable" or "unstable" mycelium or "hard" and "soft" mycelium as terms upon which separation is to depend.

The forms examined closely resemble *P. polychromogenus* (filamentous phase) and might be readily classed there. However, in view of the apparent instability of *P. polychromogenus* and the relative stability of the *P. asteroides* types here studied and in view of the more or less rare variations reported on the same types by Wright (1937), it is proposed to consider them as separate entities. *P. asteroides* is pathogenic while the pathogenicity of *P. polychromogenus* is unknown.

A readily applicable modification of the Jensen system would appear to be as follows:

Partially acid-fast types, non-proteolytic, non-diastatic, constantly utilize paraffin:
1. α mycelium types: *P. opacus*, *P. erythropolis*,
2. β mycelium types:
    a. Red colored *P. polychromogenus*, *P. asteroides*;
    b. Yellow colored *P. paraffinae*.

TYPE II. NON ACID-FAST β PROACTINOMYCES

The non acid-fast forms, possessing diastatic and proteolytic properties and unable to use paraffin as the sole source of carbon were represented in this study by 10 strains. The origin of the strains is as follows:

3072. Unidentified (N. J. Collection).
3376. *A. maculatus* Millard and Burr.
3377. *A. salmonicolor* Millard and Burr.
3308B. "*A. asteroides*," obtained from Dr. Broadhurst in 1936.
3322B. "*A. fradii*," obtained from Bucherer, Holland, in 1935.
3382,[6] 3383, 3393, 3395, 3396. (*P. flavescens.*)

The organisms here studied do not constitute an entirely homogeneous group and on casual examination would be considered as distinct and separate species. However, if the course of development is watched closely, it will be noted that the differences between them are more a matter of rate of development than of any qualitative differences between the strains. A case in point concerns the strains 3072, 3376, and 3382. On starch agar all three were identical and remained throughout their growth deep flesh-colored orange. On Czapek's synthetic medium they were identical in appearance for about five days, at which time 3376 began to show traces of black pigment until at 20 to 25 days the growth was almost entirely black except for a narrow margin of the original orange. Throughout its growth, 3072 remained orange, while 3382 showed an irregular black pigmentation (appearing among some of the replicates but not among others), which began at about the 7th to 8th day and was not complete for 30 to 40 days. Even such differences as those noted here were not constant since on continued repetition of the experiment it was noted that the black pigmentation was not a character of the strain, but seemed to be related to some other cause. For example, subcultures from the black form on the same media showed no trace of the black pigment for at least 40 days but retained its original characteristic orange; this was true of 3382, which indeed very rarely showed black pigmentation.

[6] Cultures 3382 to 3396 were isolated from Zuidersee soils and sent to Dr. Waksman for identification by Dr. Westerdijk.

These variations in pigment production are not explainable at present, but they serve to confuse any system of classification unless they are specifically noted. Further, the actual shade of the orange pigment may vary with the different strains, but the general type of pigment was easily recognized.

In table 3, the characters of the strains examined are summarized; the same remarks apply as indicated for table 2. Jensen divided the group as follows:

Non-acid-fast organisms, constantly diastatic:
1. Non-proteolytic, no aerial mycelium: *P. mesentericus;*
2. Proteolytic:
   a. Rapid growers, broth turbid *P. actinomorphus;*
   b. Slower growers, broth clear *P. flavescens.*

The same remarks apply to this separation as to the similar one for acid-fast forms and a suitable modification appears to be the following:

Non-acid-fast forms, constantly diastatic:
1. α mycelium types;
   a. Non-proteolytic *P. mesentericus,*
   b. Proteolytic *P. actinomorphus,*
2. β mycelium types:
   a. Yellow colored *P. flavescens,*
   b. Red to orange (tendency to blacken) *P. maculatus, n. sp.*

The strains here studied are included under the term *Proactinomyces maculatus* since the original *Actinomyces maculatus* strain of Millard and Burr (1926) is typical of the group. These strains are more closely related to *Actinomyces salmonicolor* M & B, but the name *Proactinomyces salmonicolor* has been preempted. *Actinomyces maculatus* M & B is described as facultatively anaerobic, though the original strain of that organism does not now possess that character, and, in fact, is indistinguishable from *Actinomyces salmonicolor.* The description of the new representative of the species is as follows:

Filamentous organisms possessing a tough shiny colony which is cartilaginous, rarely producing aerial mycelium though in certain

TABLE 3

Cultural characters of non-acid-fast β proactinomyces

NUMBER	DESCRIPTION	SYNTHETIC AGAR†										NUTRIENT AGAR										STARCH		GELATIN		MILK		PARAFFIN GROWTH	
		Growth		Color*		Aerial mycelium		Acid-fast		Fragmentation of mycelium		Growth		Color		Aerial mycelium		Acid-fast		Fragmentation of mycelium		Hydrolysis				Reaction	Acid-fast		
		Days of growth																											
		5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	5	20	30	30	5	20
3072	Unidentified	3	4	O	O-B	–	–	–	–	–	±	4	4	O-Y	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3376	*A. maculatus* M and B	2	4	O	O-B	–	–	–	–	–	±	1	2	O-W	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3377	*A. salmonicolor* M and B	3	4	O	O	–	–	–	–	–	–	3	4	O-W	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3308B	*A. asteroides*	±	±	O	O	–	–	–	–	±	±	4	4	O-B	O-B	–	±	–	–	±	±	+	+	+	+	–	–	–	–
3322B	*A. fradii*	3	4	O	O	–	–	–	–	–	±	4	4	O-B	O-R	–	–	–	–	–	±	+	+	+	+	–	–	–	–
3382	Unidentified	2	4	O	O	–	–	–	–	–	–	4	4	O	O-R	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3383	Unidentified	1	1	O	O	–	–	–	–	–	±	2	4	O-W	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3393	Unidentified	2	4	O	O-Y	+	++	–	–	–	±	2	4	O-R	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3395	Unidentified	3	4	O	O-B	–	–	–	–	–	±	4	4	O	O	–	–	–	–	–	–	+	+	+	+	–	–	–	–
3396	*P. flavoscens*	2	4	O-Y	Y	+	+	–	–	±	+	1	1	Y	Y-W	–	+	–	–	±	±	+	+	+	+	–	–	–	–
3308A	Acid-fast type (see table 2)	1	2	–	O-R	±	+	+	+	+	++	3	4	O	O	++	++	+	+	+	++	–	–	–	–	Ae	+	–	++

* O, orange; R, red; B, black; W, white; Y, yellow.
† Waksman (1919).

strains it may occur frequently. In the young colony an orange (varying from orange-yellow to orange-red) intercellular pigment is produced on all media, which may or may not change to black as the culture ages. The organisms hydrolyze starch and gelatin, do not utilize paraffin, do not digest milk, are non-acid-fast, and retain their mycelium form for long periods.

This species is designated as *Proactinomyces maculatus* (Millard and Burr) Nov. Comb. Umbreit.

Because of the variations in rates of growth and in pigment production, which has been noted earlier, further studies with representatives of the group were undertaken in an attempt to establish the factors responsible. While the attempts were unsuccessful, some of the information secured is of interest. Traces of certain metals, namely Cu, Fe, Mn, B, Mo and Va, were added to the basic medium (Czapek's sucrose-nitrate medium), in amounts equivalent to 1 mgm. of the metal per liter. The general technique in this and other growth experiments was to inoculate (usually in triplicate) a liquid medium with a suspension of the organism. After incubation (usually 2 to 3 weeks), the growth was weighed by filtering through weighed filter paper, drying at 80°C. for 24 to 48 hours and noting the increase in weight. In the case of the metals noted above the growth was never greater than that of the untreated control. Various types of extracts from manure, plants, fresh *Rhizopus* mycelium, cells of actinomycetes, germinated wheat, etc., sterilized either by heat or by filtration gave no increases in yield of cells and no indications of increasing the growth rate. The C/N ratio of the medium was varied without influencing the growth until limiting amounts of either nitrate or sucrose were reached. Since it was noted that growth in general seemed better on agar media than on the corresponding liquid media and since additions of small amounts of agar had no effect, it was thought possible that the surface available might be a factor in growth. Several experiments were conducted using glass beads, washed sand, asbestos and glass slides to provide surface. While occasionally an increase in growth was obtained by these measures, the results were inconsistent and seemed to point to surface as operating

indirectly through perhaps the O/R potential. Additions of reducing agents inhibited growth (cysteine and reduced iron were used) but in liquid media additions of potassium permanganate (final concentration M/100, M/1000) increased the rate of growth slightly. Aeration of the media with air inhibited growth, so that it was considered that free oxygen, high O/R potential, and surface (at least in an undisturbed medium) were necessary. Shake cultures using glucose or starch as an energy source with additions of oxidizing agents ($KMnO_4$, M/100, M/1000, M/10,000 final concentration; $K_3Fe(CN)_6$ 1/100, 1/1000/0; Fe citrate 1/10, 1/100, 1/1000) yielded growth only at the surface, and the actual amount of growth did not differ significantly from the controls. These results emphasize the necessity of free oxygen.

Various nitrogen sources were also studied. The compounds listed below were added in amounts to give 0.3 gram N/liter. $NaNO_3$ was the best source of nitrogen, followed by glutamic acid, egg albumin, zein, urea, leucine, dried blood, aspartic acid, alanine, ammonium sulfate, ammonium lactate, tyrosine, phenylalanine, casein, in the order given. No growth was obtained (strain 3382) on acetamide, caffein, l-cystine, hippuric acid, or keratin and only traces on glycine. In all the experiments reported here the organism maintained its original orange pigment so that no information was gained as to the cause of blackening. The general course of development of the strains in this group may be briefly described as follows:

On Czapek's agar the strains show pigmentation, in from 2–5 days, on other media usually within 3 days; the pigment is usually flesh color with no trace of black. The mycelium remains without appreciable fragmentation for 20 to 30 days. Aerial mycelium is seldom present, and if present is likely to appear within the first ten days. For some unknown reason, occasionally a sub-culture will grow very slowly while another will grow rapidly. This could not be related to any factor except the amount of inoculum, which is difficult to control.

For groups of organisms where such inconsistent differences appear, it is necessary to study the entire course of development over a period of time, to include several replicates, and to repeat

the studies periodically. Culture 3396 seems to resemble *Proactinomyces flavescens* though physiologically it is indistinguishable from the others.

SUMMARY AND CONCLUSIONS

An examination of several well-defined species and many unidentified organisms belonging to *Proactinomyces* of Jensen has shown that:

1. The criteria for separation of the group from *Actinomyces* proper (the absence of spore formation) is a valid one.
2. Because of the difficulty of determining and defining mycelium formation, the lower limits of the group are not adequately distinguishable from the closely related *Corynebacterium* or *Mycobacterium.*
3. Within the *Proactinomyces* two groups are distinguishable. These have been separated as follows: (a) α *Proactinomyces,* characterized by short unstable mycelium, "soft" bacteria-like colony, diffused bacterial growth in liquid media. (b) β *Proactinomyces,* characterized by long stable mycelium, "hard" Actinomyces-like colony, typical Actinomyces (colony) growth in liquid media. The validity of the diagnostic features is discussed in detail in the text.
4. The "stable" mycelium type (β) was studied more closely and it is shown that a further separation on the basis of partial acid-fastness is possible. Slight modifications of the present separations appear desirable and a further description and emendation of the species *Actinomyces maculatus* Millard & Burr is given. A transfer of this species to the genus *Proactinomyces* is proposed; e.g. *Proactinomyces maculatus* (Millard & Burr) Nov. Comb. Umbreit.

The author takes this opportunity of expressing his indebtedness to the several workers who aided materially in the work through contributions of cultures or criticisms of the manuscript, and especially to Professor S. A. Waksman without whose continual guidance little could have been accomplished.

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SOME SEROLOGICAL RELATIONSHIPS OF THE S, R, AND G PHASES OF BACILLUS SALMONICIDA[1]

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The cultural and pathogenic characteristics of British Columbia strains of *Bacillus salmonicida* have been recorded (Duff and Stewart, 1933), and the S, R, and G variants of the microörganism were later described (Duff, 1937). The present report deals with the application of reciprocal agglutinin absorption methods in an attempt to discover the serological connections between the S, R, and G phases. The work has been designed to elicit both the relationships existing between the variants recovered from individual strains, and also those existing between the variants of different strains.

A total of 19 British Columbia strains have been examined. Of these, 8 have been subjected to full reciprocal tests (Krumwiede, Cooper and Provost, 1925); the remainder have been tested in respect only to their agglutinability by suitably absorbed antisera.

EXPERIMENTAL

Origin of variants

The S, R, and G variants were obtained by subjecting stock cultures (usually R) to serial growth in 0.25 per cent lithium chloride broth (Hadley, Delves and Klimek, 1931; Duff, 1937). The colonial distinction between the R and S dissociants was found to be most marked when platings from the broth series were made upon peptone-meat extract medium containing 1.5 per cent sodium chloride and 0.8 per cent agar. G-phase cultures

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were best recovered from platings on a medium containing the same nutrient base plus 0.5 per cent sodium chloride and 1.5 per cent agar. The use of two media for plating was adopted solely in order to facilitate rapid isolation. It should be noted in this connection that all three dissociants are culturally distinct upon any one of the usual laboratory media (Plate I).

Antigen and antiserum production

Immediately prior to antigen production, single-cell cultures of the S and R variants were obtained, only 2 transfers intervening between the growth from the single cell and the mass antigen culture. All antigens were grown on nutrient agar at approximately 22°C., washed off in saline suspension, and preserved with 0.2 per cent formaldehyde. S and R cultures were harvested at 48 hours, G cultures at 6 days. Precaution was taken to obviate the presence of agar particles in the preparations. Antisera were prepared by inoculation of rabbits by the intravenous route.

Salt-stability

Blake and Anderson (1930) considered saline suspensions of *B. salmonicida* to be so susceptible of spontaneous flocculation that they could not apply agglutination methods in the serological examination of the organism. With 19 British Columbia strains of the organism we have consistently obtained stable suspensions of all pure S and G phase cultures in 0.8 per cent sodium chloride. The R phase cultures, while readily auto-agglutinable in salines down to 0.2 per cent, form satisfactorily stable suspensions in 0.05 per cent sodium chloride. This concentration of electrolyte appears still sufficient to permit completion of specific agglutination, since the final titre of an S strain in 0.05 per cent saline is the same as its titre in 0.8 per cent saline, when titration in both cases is carried out by incubation at 37°C. for 4 hours, followed by overnight storage in the ice-box. All S G-phase suspensions employed were peculiar in behaving in a manner the reverse of R suspensions (table 1). They were therefore always titrated in 0.8 per cent saline.

Absorbing doses

Minimal absorbing doses for S and G cultures were always between 1:10 and 1:5 (volume of packed cells: 1/10 antiserum), whereas the value for R cultures was never less than 1:2. This observation is in agreement with those of Teague and McWilliams (1917) and of Krumwiede, Cooper and Provost (1935), who noted that the R or spontaneously-sedimenting strains of various bacterial species showed an agglutinin-absorbing capacity considerably lower than that of corresponding S strains.

Reciprocal absorptions between variants within single strains

The technique followed with all sera was that of absorption with a low multiple of the minimal absorbing dose, followed by

TABLE 1

Salt stability of variants of B. salmonicida

STRAIN	PHASE	FLOCCULATION IN DISTILLED WATER	FLOCCULATION IN NaCl CONCENTRATIONS OF (PER CENT)				
			0.05	0.1	0.25	0.5	0.85
SP 2............	Stock, chiefly R	–	–	tr.	+	2+	2+
SP 2............	R	–	–	+	3+	4+	4+
SP 2............	S	–	–	–	–	–	–
MGS 7..........	G (unstable)	?	?	+	–	–	–
GS 5............	G (stable)	3+	2+	2+	–	–	–

titration of the absorbed serum. Absorptions were carried out for 4 hours at 37°C. followed by icebox storage overnight. Table 2 illustrates a typical result with *B. salmonicida* strain CL 6.

Considering first the R and S interconnections, five strains, CL 6, CL 11, SP 5, SP 13, ER 2, were found to possess essentially the same antigenic relationship between the R and S phase cultures as has been illustrated for strain CL 6. In these strains the R cells of any given strain appear to possess an antigen or antigens not present in S cells of the same strain. The antigenic situation may be represented in symbols as, R = S + n. For convenience *B. salmonicida* strains so constituted may be termed Group I strains. This relationship was not however found to

hold for certain other strains. Three strains, CL 3, CL 4, SP 9 (Group II) displayed a different antigenic picture, in which the

TABLE 2

Reciprocal relationships of the R, S, and G variants of B. salmonicida, strain CL 6

ABSORPTION: ANTISERUM	ABSORPTION: CELLS	CELL SUSPENSION FOR TITRATION	DILUTIONS OF ABSORBED SERUM: 1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	C
R	S	R	3+	3+	3+	2+	–	–	–	–	–
S	R	S	–	–	–	–	–	–	–	–	–
R	G	R	2+	2+	2+	+	–	–	–	–	–
G	R	G	3+	2+	+	–	–	–	–	–	–
S	G	S	+	2+	2+	2+	–	–	–	–	–
G	S	G	3+	2+	+	–	–	–	–	–	–
R	R	R	–	–	–	–	–	–	–	–	–
S	S	S	–	–	–	–	–	–	–	–	–
G	G	G	–	–	–	–	–	–	–	–	–
R (unabsorbed)		R	4+	4+	4+	3+	2+	2+	+	+	–
S (unabsorbed)		S	4+	3+	2+	2+	2+	2+	2+	–	–
G (unabsorbed)		G	4+	3+	3+	2+	2+	+	+	–	–

TABLE 3

Reciprocal relationships of the R, S, and G variants of B. salmonicida, strain CL 3

ABSORPTION: ANTISERUM	ABSORPTION: CELLS	CELL SUSPENSION FOR TITRATION	DILUTIONS OF ABSORBED SERUM: 1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	C
R	S	R	–	–	–	–	–	–	–	–	–	–
S	R	S	3+	3+	3+	3+	2+	–	–	–	–	–
R	G	R	3+	3+	3+	3+	2+	–	–	–	–	–
G	R	G	3+	3+	3+	3+	2+	2+	2+	–	–	–
S	G	S	2+	2+	+	+	–	–	–	–	–	–
G	S	G	2+	3+	3+	3+	2+	2+	2+	–	–	–
R	R	R	–	–	–	–	–	–	–	–	–	–
S	S	S	–	–	–	–	–	–	–	–	–	–
G	G	G	–	–	–	–	–	–	–	–	–	–
R (unabsorbed)		R	4+	3+	3+	4+	4+	4+	4+	2+	2+	–
S (unabsorbed)		S	4+	4+	3+	3+	3+	3+	3+	2+	+	–
G (unabsorbed)		G	4+	4+	4+	4+	4+	3+	3+	2+	–	–

S cells possessed an antigen or antigens not present in the corresponding R cells, or, $S_1 = R_1 + n_1$. A summary of the absorption findings for Group II strain CL 3 appears in table 3.

Typing of additional strains

It proved impracticable to extend full reciprocal analysis to further individual strains of *Bacillus salmonicida.* At the same time it was of definite interest to know whether other strains would all fall into one or other of the serological groups (in respect of the R and S relations) so far established for an admittedly small number of strains. A tentative method for testing new strains was therefore devised. The method is based on an observation on the relationship existing between the "n" antigen complex of Group I strains (found only in R phase cells) and the "n_1" complex of Group II strains (found only in S phase cells). Experiments leading to this observation had been designed to discover whether the "n" antigen of the R cells of strain CL 6 was shared by the R cells of the remaining strains of Group I. That is, while the relationship R = S + n had been shown to hold for any given strain in Group I, it had not yet been demonstrated whether the antigen labelled "n" differed in nature from strain to strain within the group, or whether it remained antigenically identical in all 5 strains. There was also the question as to the antigenic similarity, or lack of similarity, between the n antigen of the R cells of Group I strains and the n_1 antigen of the S cells of Group II strains.

It will be seen that absorption, using homologous S cells, of an antiserum made against the R cells of any one strain selected from Group I yields what may be termed a "monovalent n" antiserum. A serum of this character was prepared by absorbing an antiserum made against R phase cells of strain CL 6, with S phase cells of the same strain. The absorption was carried out twice, each time using 2 times the minimal absorbing dose of S cells. (The minimal absorbing dose was 1:2, therefore 1 part 1:9 immune serum was added to 1 part of packed cells, mixed, allowed to absorb, then centrifugalized. The supernatant was withdrawn and again added to an equal volume of packed cells. Absorption and centrifugalization followed, giving a twice-absorbed antiserum of approximately 1:10 dilution.)

Table 4 records the agglutinability of all strains under the

influence of the CL 6 monovalent n antiserum. These results do not necessarily prove the complete identity of the CL 6 n antigen with the n antigen of the remaining Group I strains or with the n_1 antigens of the three GroupI I strains. They do, however, demonstrate that this antigen is common to the R cells only of all Group I strains and to the S cells only of all Group II strains. This finding would therefore seem to act not only as a check on the validity of creating the two antigenic groups, but also as an extension of the fundamental significance of the grouping.

TABLE 4

Agglutination of R and S phases of group I and group II strains by CL 6 monovalent N serum

PHASE	GROUP I					GROUP II		
	CL 6	CL 11	SP 5	SP 13	ER 2	CL 3	CL 4	SP 9
R.........	160*	160	80	160	80	—	—	—
S.........	—	?	—	—	—	80	40	80

* Figures represent the highest serum dilution giving 2+ agglutination after 37°C. incubation for 4 hours, read after overnight icebox storage.

TABLE 5

Grouping of B. salmonicida strains by means of agglutination with monovalent N serum

PHASE	CL 7	CL 8	CL 10	SP 1	SP 3	SP 4	SP 12	SP 19	SP 22	ER 1	ER 3
R..............	160*	80	—	80	—	160	160	40	80	160	160
S....	—	—	160	—	320	—	—	—	—	—	—
Group..	I	I	II	I	II	I	I	I	I	I	I

* As for table 4.

On the basis of these findings, a number of other *B. salmonicida* strains were tested in respect of the agglutinability of their R and S phase cells by CL 6 monovalent n serum. The R and S variants were obtained from 11 strains by the methods already described. Their reaction to the absorbed serum is presented in table 5. It will be noted that in all cases, agglutination occurred in either the R cell suspension or in the S cell suspension, but never in both. The method of grouping therefore remained valid for these strains.

Antigenic relationship of the G variant to R and S variants

The G phase cultures examined comprise only those derived from *B. salmonicida* strains Nos. CL 3, CL 4, CL 6, CL 11, SP 5, SP 9, SP 13, ER 2. Reciprocal absorptions were carried out using G cells and G antiserum against cells and serum of the corresponding R and S phases. With all strains, the so-called partial-partial absorption presented itself. Typical examples are given in tables 2 and 3. This type of absorption result might be taken to mean that the G phase cell is composed in part

TABLE 6

Reciprocal absorption between G phases of 3 strains of B. salmonicida

Absorption: Antiserum	Absorption: Cells	Cell suspension for titration	Dilutions of absorbed serum: 1:40	1:80	1:160	1:320	1:640	1:1780	1:2560	1:5120	C
G-CL 6	G-CL 6.......	G-CL 6	−	−	−	−	−	−	−	−	−
	G-CL 3.......	G-CL 6	2+	+	−	−	−	−	−	−	−
	G-SP 2.......	G-CL 6	2+	+	+	−	−	−	−	−	−
G-CL 3	G-CL 3.......	G-CL 3	−	−	−	−	−	−	−	−	−
	G-CL 6.......	G-CL 3	−	−	−	−	−	−	−	−	−
	G-SP 2.......	G-CL 3	−	−	−	−	−	−	−	−	−
G-SP 2	G-SP 2........	G-SP 2	−	−	−	−	−	−	−	−	−
	G-CL 6.......	G-SP 2	2+	+	−	−	−	−	−	−	−
	G-CL 3.......	G-SP 2	2+	2+	+	−	−	−	−	−	−
G-CL 6 (unabsorbed)...		G-CL 6	4+	3+	3+	2+	2+	+	+	−	−
G-CL 3 (unabsorbed)...		G-CL 3	2+	3+	3+	3+	2+	2+	+	+	−
G-SP 2 (unabsorbed)...		G-SP 2	4+	4+	3+	2+	2+	2+	+	−	−

of an individual G antigen or antigen complex, and in part of an antigen or antigens common to itself and to the R and S cell of the same strain. There is an alternative possibility which will be referred to later.

Reciprocal absorptions were further carried out between the G phases of strains CL 3, CL 6, and SP 2. The results, recorded in table 6, show a high degree of antigenic similarity.

DISCUSSION

It must be remembered that the absorption technique employed in these experiments serves only to discover qualitative antigenic

differences between the culture variants in question. The use of multiples of the minimal absorbing dose enables one to say that two cultures possess (or do not possess) a particular antigen in common. One is not able however to gain knowledge as to the relative amount of a common antigen which is shared by two or more cultures. For instance, in Group I *B. salmonicida* strains, no attempt can be made to say what proportion of the R cell consists of S antigen. Problems of this type are discussed by Krumwiede, Cooper and Provost (1925) in relation to the antigenic constitution of variants of a single species. Wilson and Miles (1932) developed a successful technique in connection with the differentiation of smooth strains of *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*, all of which appear to possess identical antigens but in varying proportions. Work of this nature does not come within the scope of the present report.

Due to the comparatively small number of individual cultures examined, no claim is made that all strains of *B. salmonicida* will necessarily fall into two serological groups, in respect to the location of the n antigen in the R or in the S phase of the strain under consideration. It is, however, interesting that the 19 strains so typed were selected at random from 45 individual strains of the organism, all of which were obtained from different fish from three different localities. The origin of the culture did not appear to be a deciding factor in this method of grouping. All CL strains were obtained from an epizootic at Cultus Lake, British Columbia. Of these, 4 fell into Group I, 3 into Group II. All SP strains originated in Stanley Park, Vancouver, and of these 7 typed as Group I and 2 as Group II. The three ER (Elk River, B.C.) strains all typed as Group I; this could obviously be a matter of chance.

In interpreting the results of the reciprocal absorptions of G variants with the homologous R and S cultures, Dubos (1938) has suggested that it does not necessarily follow that the G cells possess an individual antigen not shared by the corresponding R and S cells. One could alternatively postulate a "masking" condition in the R and S cells, so that an antigen common to all three variants, possibly of a deep somatic nature, would be over-

laid in the R and S cells by antigens situated on or near the surface. This point is of considerable interest, and work based on Dubos' methods of controlled hydrolysis has been planned to elucidate the situation.

The antigenic picture brought out by the absorption methods described fails to account for the difference in pathogenicity of the variants. Culturally true S variants of both Groups I and II are, or can readily become, pathogenic. All R and G variants are without exception non-pathogenic.

SUMMARY

1. Examination of the antigenic relationship of the S, R, and G variants of 8 strains of *Bacillus salmonicida* has been made by the method of reciprocal agglutinin absorption. In 5 strains (Group I) the R and S relationship may be briefly presented in the form $R = S + n$, and in 3 strains (Group II) in the form $S_1 = R_1 + n_1$.

2. The n antigen of R cells of strain CL 6 is present in the R phase only of Group I strains, and in the S phase only of Group II strains.

3. A further 11 strains were typed on the basis of the agglutinability of their S or R phases by a monovalent absorbed serum containing antibody to the n antigen of strain CL 6. Nine strains showed agglutination of R cells only and were thus assigned to Group I. Three strains showed agglutination of S cells only, and were placed in Group II.

4. Pathogenicity is not correlated with the antigenic picture elicited by the absorption methods used in these experiments.

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PLATE 1

Fig. 1. *B. salmonicida*, 24 hours; R colonies white, S colonies gray.

Figs. 2 and 3. *B. salmonicida*, 48 hours; R colonies white, S colonies gray.

Fig. 4. G colonies, 6 days.

All photographs × 1; oblique transmitted white light with red filter on camera

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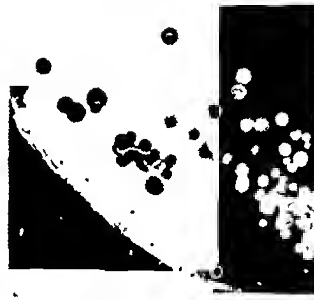

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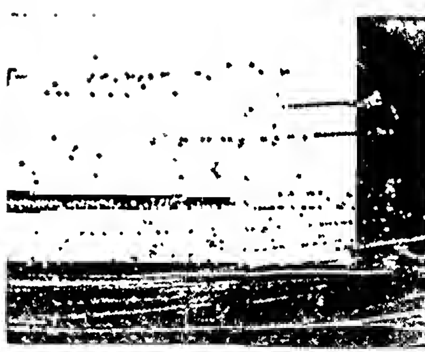

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(D. C. B. Duff. Serological Relationships of B salmonicida)

LES CILS CHEZ LES BACILLES APPARTENANT AU GROUPE DES FUSIFORMIS

W. N. KAZEEFF

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Parmi les trois bacilles appartenant au groupe des *fusiformis: Fusiformis dentium Hoelling, Fusiformis nucleatus* et *Fusiformis polymorphus*, c'est le *Fusiformis dentium Hoelling* qui intéresse surtout les bactériologistes à cause de son pouvoir pathogène dans l'angine de Vincent et dans d'autres infections.

Les avis des bactériologistes sur la mobilité et en conséquence sur la présence de cils chez ce bacille se trouvant partagés, nous avons étudié plusieurs prélévements directs de l'angine de Vincent provenant de divers malades.

Aucun de ces prélévements directs, émulsionné immédiatement et coloré ensuite par des réactifs même propres à la coloration de cils ne nous a montré les *Fusiformis dentium Hoelling* munis des cils.

Un jour ayant effectué le prélévement d'une angine de Vincent et manquant de lames rigoureusement propres, l'idée nous est venue d'essayer de conserver le prélévement en question dans des conditions suivantes. Nous avons mis 4 ou 5 gouttes de sérum physiologique dans un tube stérile dans lequel nous avons placé l'écouvillon portant le prélévement et après avoir bouché avec du coton et capuchonné le tube nous l'avons placé à l'étuve à 37° pendant 15 heures. Ensuite nous avons porté directement une parcelle du prélévement sur une lame rigoureusement propre par contact du coton d'écouvillon. Cette parcelle étant ensuite immédiatement étalée comme un frottis de sang nous avons procédé à la coloration par la méthode classique de Tribondeau-Fontana (mordançage à chaud par le tanin à 5%, et coloration à chaud par la solution de Fontana contenant 5% de nitrate d'argent).

L'examen au microscope des lames ainsi colorées nous a montré à côté des *Borrelia vincenti* de nombreux *Fusiformis dentium Hoelling* munis des cils peritriches, comme le montrent nos microphotographies. Ces cils ont un tout autre aspect et, comme nous le supposons, une toute autre constitution que les cils propres à la plupart de microbes tant aérobies que anaérobies. Sur chaque *Fusiformis dentium Hoelling* cilié on rencontre généralement un certain nombre de cils plus épais à côté d'autres cils moins épais. Ces gros cils beaucoup plus épais près du corps du bacille s'effilent de plus en plus pour se terminer par un bout acéré. Tous ces cils surtout ceux plus épais sont moins flexueux que ne le sont généralement les cils bactériens et par leur aspect il ressemblent aux tentacules d'un poulpe plutôt qu'aux cils baetériens. D'ailleurs le fait qu'ils prennent suffisamment la coloration par le réaetif de Tribondeau-Fontana pour lequel tous les cils bactériens communs restent réfractaires nous semble parler en eette faveur.

Nous ne nous sommes pas préoccupés spécialement ni de *Fusiformis nucleatus* ni de celui *Fusiformis polymorphus*. Cependant eomme ees deux bacilles se trouvent toujours comme saprophytes dans toute cavité bueeale, nous les avons rencontrés tant dans des prélévements de l'angine de Vincent que dans ceux de la eavité orale saine.

Fusiformis nucleatus. Ce saprophyte vulgaire de la cavité buccale qu'on reneontre inévitablement dans toute salive possède aussi des cils peritriches. Coloré par le procédé de Tribondeau-Fontana, il se présente généralement sous l'aspect d'un petit bâtonnet munis d'un cil soit terminal, soit latéral (fig. 2 et 4). Cependant on peut reneontrer rarement ce bacille portant 2, 3, 4, et même plus de cils analogues à ceux de *Fusiformis dentium Hoelling.*

Disons en passant que nous avons reneontré ce bacille toujours cilié dans des excréments humains colorés par le procédé propre à la coloration de cils bactériens.

Fusiformis polymorphus. Bien que nous ayons rencontré parfois sur nos préparations des bâtonnets fusiformes dont la longueur dépassait celle de *Fusiformis dentium Hoelling* et portant

des cils pareils comme chez ce dernier (fig. 5), nous nous abstenons, par prudence, d'affirmer s'il s'agissait vraiment d'un *Fusiformis polymorphus* ou d'un assemblement de deux *Fusiformis dentium Hoelling*.

CONCLUSIONS

Le *Fusiformis dentium Hoelling* et le *Fusiformis nucleatus* sont munis des cils peritriches; ils sont donc en conséquence mobiles. Leurs cils se colorent à l'encontre des cils communs bactériens par la méthode de Tribondeau-Fontana. Ce fait ainsi que leur aspect microscopique permettent de supposer que ces cils sont d'une constitution autre que celle de cils communs bactériens.

Quant au *Fusiformis polymorphus*, bien que certains indices permettent le considérer également comme cilié, aucune preuve irréfutable n'est recueillie par nous pour maintenir cette affirmation.

PLATE 1

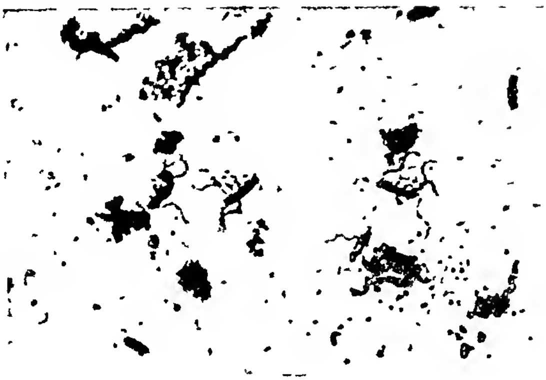
FIG. 1. QUATRE FUSIFORMIS DENTIUM HOELLING PARMI BORRELIA VINCENTI

FIG. 2. FUSIFORMIS DENTIUM HOELLING (EN BAS ET À GAUCHE) ET BAC. FUSIFORMIS NUCLEATUS (EN HAUT ET À DROITE)

FIG. 3. FUSIFORMIS DENTIUM HOELLING ET BORRELIA VINCENTI

FIG. 4. TROIS FUSIFORMIS DENTIUM HOELLING ET UN BAC. FUSIFORMIS-NUCLEATUS (PLUS PETIT PORTANT UN CIL LATERAL)

FIG. 5. CINQ FUSIFORMIS DENTIUM HOELLING PARMI BORRELIA VINCENTI
Le sixième gros bâtonnet cilié (au milieu et en bas) semble être Fusiformis polymorphus.



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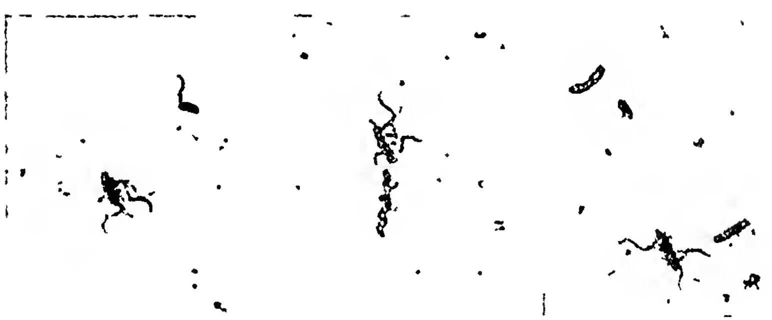

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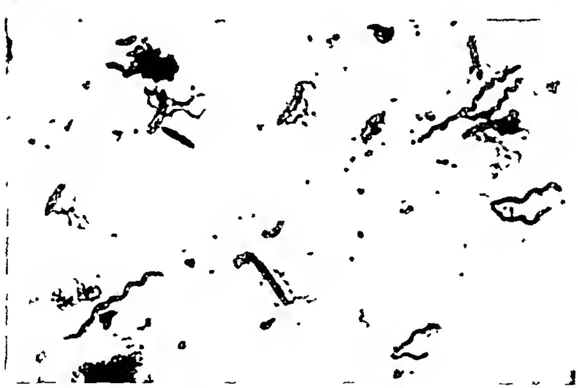

5

(W. N. Kazeeff Les cils chez les bacilles fusiformis)

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH

NINTH ANNUAL MEETING, IOWA STATE COLLEGE, AMES, IOWA, MAY 5-6, 1939

STUDIES ON A PRESUMABLY NON-PATHOGENIC, ACID-FAST MICROORGANISM FREQUENTLY PRESENT IN THE TONSILLAR TISSUE OF SWINE. *A. G. Karlson and Wm. H. Feldman*, The Mayo Foundation, Rochester, Minnesota.

One tonsil from each of 47 swine with gross lesions of tuberculosis was examined; fourteen yielded avian tubercle bacilli. From thirteen, a rapidly-growing saprophytic acid-fast microorganism was isolated. A similar number of tonsils of grossly non-tuberculous swine yielded avian tubercle bacilli in eight cases; saprophytes were recovered from eleven.

The studies indicate that the twenty-four strains of saprophytic acid-fast bacilli are identical. They develop rapidly on glycerinated mediums forming a pasty, gray or cream-colored, rough growth. On liquid mediums a heavy crinkled pellicle which finally sinks is formed. They do not use glucose, lactose, sucrose, sorbitol, mannitol, arabinose, levulose, trehelose or galactose as the sole carbon source on Merrill's medium. Glycerol is utilized with acid formation. Optimum growth occurs at 38°C. No growth occurs at 47°C. They fail to survive an exposure to 60°C. for an hour.

They produce no demonstrable disease in calves, chickens, rabbits or mice. In these animals no sensitivity to tuberculin or homologous culture filtrates was detected. Guinea pigs infrequently developed an abscess following subcutaneous injection of large doses and a transitory sensitivity to avian tuberculin and homologous culture filtrates was demonstrated. Agglutination studies indicate that the strains are antigenically alike and that they have antigens in common with *Mycobacterium avium* and *Mycobacterium phlei*.

CARBOHYDRATE METABOLISM OF AEROBACILLUS SPECIES—A PRELIMINARY REPORT. *R. W. H. Gillespie*, Assistant Professor of Bacteriology University of South Dakota.

A study was made of the degradation of glucose by *Aerobacillus maccrans* and *Aerobacillus polymyxa*. Cultures of several strains of each species were incubated, aerobically and anaerobically, for 4 to 5 days at 37°C. in 1.0% and 1.5% glucose extract broth. The cultures were then analysed quantitatively for residual reducing sugar, for volatile and non-volatile acids, and for alcohol.

A. polymyxa appears to utilize glucose more rapidly and completely than *A. macerans*. Volatile and non-volatile acids appear to accumulate more extensively in the *A. maccrans* cultures. Alcohol was found in larger quantities in the *A. polymyxa* cultures.

Thus, the results indicate that the

degradation of glucose proceeds differently, in rate, at least, in cultures of the two species. An attempt is to be made to correlate differences in carbohydrate metabolism with previously observed differences in oxidation-reduction potential which characterize the species.

Respiratory Factor for Rhizobium. *R. H. Burris and P. W. Wilson,* University of Wisconsin.

Adsorption of Sulfanilamide in the Presence of Peptone. *Robert E. Hoyt and Milton Levine,* University of Minnesota.

A Division of the Alfalfa Cross-inoculation Group Correlating Efficiency in Nitrogen-fixation with Source of Rhizobium meliloti. *J. C. Burton and L. W. Erdman,* The Nitragin Company.

Effect of Working Butter on the Growth of Bacteria in It. *H. F. Long and B. W. Hammer,* Iowa State College.

The effect of working butter on the growth of bacteria has been reported previously. As judged by the rate of growth, time of appearance of defects, changes in pH of butter serum and changes in the acidity of fat of butter, organisms are more active in underworked than in thoroughly worked butter.

Recently the effect of reworking butter on the growth of bacteria was studied in an attempt to explain the deterioration that sometimes occurs under commercial conditions when butter is reworked or when it is printed in a special type of butter printer. Portions of under worked and moderately worked butter made experimentally from pasteurized cream inoculated with various pure cultures of organisms were held several days at approximately 10°C. before reworking. In reworked butter the organisms increased more rapidly and defects appeared more quickly than in the control butter which was not reworked. In reworked butter containing butter culture the pH of the serum decreased more rapidly and reached lower final values than in the control butter while in reworked butter containing a lipolytic organism acid numbers of the fat were higher than in control butter which was not reworked.

Bacteriostasis Due to Sulfapyridine. *Robert E. Hoyt, Kenneth J. Johnson and Milton Levine,* University of Minnesota.

Effect of Biotin Concentrates on Growth of Rhizobium and Related Species. *P. M. West and P. W. Wilson,* University of Wisconsin.

Biotin concentrates were found capable, at three hundredths of a gamma per ml., of replacing the stimulative effect of yeast extract on the growth of *Rhizobium trifolii* 205. A survey of the effect of this preparation on various species of *Rhizobium* and related forms was made to determine whether this same growth factor was required in all cases for maximum development. All "fast-growers" responded to the biotin concentrate with the exception of a few strains which grew well without the factor and no better in its presence. *Rhizobium lupini* responded well, but no strains of "slow-growers" of the soybean or cowpea groups showed stimulation even if high levels of biotin concentrate were added. *Phytomonas tumefaciens, Achromobacter radiobacter* and two species of *Azotobacter* grew well in the carbohydrate mineral salts base medium and remained unaffected

by biotin additions. "Coenzyme R" preparations tested in place of biotin concentrates at fifty gammas per ml. gave similar results throughout. *Azotobacter*, due to its ability to synthesize the growth factor for *R. trifolii* 205 showed no response to the biotin concentrate.

Note on the Preparation of Active Cell-free Juice from Bacteria. *W. P. Wiggert, M. Silverman, M. F. Utter and C. H. Werkman*, Bacteriology Section, Industrial Science Research Institute, Iowa State College, Ames.

An active cell-free juice which attacked hexosediphosphate and hexosediphosphate plus glucose anaerobically was obtained from *Aerobacter indologenes* under the following conditions: 3 grams of cell paste, autolyzed 24–30 hours were mixed with 25 grams ground Pyrex glass (ground 24 hours in ball mill) and 7 ml. of M/15 phosphate buffer (pH 7.0). 10 grams of this mixture were ground 5 minutes by hand in an iced mortar and the mixture extracted with 2 ml. of M/15 phosphate buffer (pH 6.6) and clarified on a Beam ultracentrifuge. The supernate showed activity. Juice has been found active after two weeks' storage in a frozen state. Several dehydrogenases were found present by the methylene blue technique.

Immunity in Canine Oral Papillomatosis. *R. J. Goodlow*, Department of Bacteriology and Immunology, University of Minnesota.

Virus-induced papillomas of the oral mucosa of young dogs regress spontaneously, leaving the animal resistant to reinfection. That regression is accompanied by development of specific antibodies in the blood serum of infected puppies is evidenced by a positive complement-fixation test.

To 1.5 ml. of saline was added 0.1 ml. of a 0.33 per cent saline suspension of emulsified tumor tissue, and one drop of blood serum of puppies on which papillomas had grown and regressed. This system was placed in the refrigerator for one hour before the addition of 2 units of complement (titrated before each test). After incubation at 37°C. for one hour the hemolytic system was added. Test readings were taken after 45 minutes of incubation. To eliminate the possibility of false positive reactions, the serum and antigen were tested before use for anticomplementary effect.

The blood sera of eight dogs infected with papillomas which had regressed were tested. In all cases complete inhibition of hemolysis, which indicates the presence of specific antibodies, was noted. Negative reactions were obtained with sera from eight normal dogs. The serum of rabbits hyperimmunized to the Shope papilloma virus did not possess the ability to fix complement in the presence of the antigen of canine oral papillomatosis.

Preparation of Bacteriological Peptones. *Einar Leifson*, Department of Bacteriology, University of South Dakota, and *Ben Diamond*, South Dakota State Health Laboratory, Vermillion, South Dakota.

The need for bacteriological peptones made according to detailed published procedures is too obvious to require any further comment. Work was started by the senior author at the Johns Hopkins University some three years ago to remedy this situation. One hundred and fifteen peptones were made, dried, and powdered. The substrates used included beef, beef heart,

beef spleen, beef lung, pork, hog stomach, fish, casein, wheat gluten, and soybean flour. The enzymes used, except papain, were prepared in the laboratory and included pepsin, pancreatin, papain, as well as serial digestions with combinations of these enzymes. Work is in progress with additional substrates. These peptones, in addition to a number of commercial peptones, have been tested extensively for diphtheria-toxin production, growth-promoting properties for some 50 selected strains of bacteria, and suitability for various biochemical tests such as indol, M.R., V.P., double-zone formation by streptococci in blood agar, pigment and gas production. The results are too voluminous to permit even a sketchy summary. The data indicate that peptones as good or better than the present commercial products may be made cheaply and with great ease. Many of the peptones produce high concentrations of diphtheria toxin. The authors invite correspondence, and would welcome cooperation in the making of special tests on the peptones.

Modification of Distemper Virus by Animal Passage. *R. G. Green* Department of Bacteriology and Immunology, University of Minnesota.

Strains of distemper virus isolated from various species of the families *Mustelidae* and *Canidae* are found, on a basis of general characters and cross immunity tests, to be identical. The cytoplasmic and intranuclear inclusions typical of the distemper virus are seen in all species of these groups. Viruses isolated from naturally infected animals show marked pathogenic properties in such diverse species as the ferret and the dog. Fifty serial transfers of the distemper virus through ferrets result in a virus highly pathogenic for ferrets and related animals but only slightly pathogenic for dogs and foxes. Susceptibility tests and serial transmissions of virus through other species of the family *Mustelidae* show that passage through members of the family *Mustelidae* increases the pathogenicity of the virus for species of that family and decreases the severity of the infection for species of the family *Canidae*. The reciprocal relationship also seems true: passage of the virus serially through members of the family *Canidae* decreases its virulence for species of the family *Mustelidae*. The mild infections produced by such modified viruses immunized against subsequent infections with strains of the distemper virus that are highly pathogenic for the species tested in this manner.

Amino Acid Requirements of the Heterofermentative Lactic Acid Bacteria. *H. G. Wood, Charles Geiger and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment State, Ames.

The amino-acid requirements of three species of heterofermentative lactic acid bacteria L2, *Lactobacillus mannitopoeus;* L4, *Lactobacillus buchneri;* L5, *Lactobacillus lycopersici* were determined on the basis of optimum acid production in a basal medium containing glucose, thiamin, riboflavin, sodium acetate and inorganic salts including ammonium sulfate. Nineteen amino-acids, those present in hydrolyzed casein with the exception of hydroxyglutamic acid, were used in the investigation. When all nineteen amino acids were added to the medium growth was luxuriant. When each amino-acid was omitted singly and the remaining eighteen added, acid production and growth was retarded in each case with the exception of glycine,

proline, hydroxyproline, leucine and isoleucine, also tryptophane for culture L4. When both leucine and isoleucine were removed acid production was not optimum. Leucine and isoleucine are replaceable, i.e. one of the two is needed but not both. The group consisting of glycine, proline, hydroxyproline and either leucine or isoleucine could be omitted with little change in growth. The remaining fifteen amino acids, alanine, valine, glutamic acid, aspartic acid, phenylalanine, tyrosine, threonine, methionine, tryptophane (L4 an exception), cystine, serine, arginine, lysine and histidine are influential on acid production, though histidine is less effective than the others. This is the first case which has come to the authors' attention in which threonine is needed by bacteria.

PRODUCTION OF TRIMETHYLENEGLYCOL BY AEROBACTER AEROGENES. *M. N. Mickelson and C. H. Werkman*, Bacteriology Section, Industrial Science Research Institute, Iowa State College, Ames.

Contrary to previous investigators, *Aerobacter* has been found to produce large yields of trimethyleneglycol from glycerol under anaerobic conditions in a glycerol mineral medium. Two strains of *Aerobacter aerogenes* and two unidentified species of *Aerobacter* were used. Yields of trimethyleneglycol in the neighborhood of 45 per cent of the fermented glycerol were obtained. Small amounts of acetylmethylcarbinol and considerable 2,3-butylene glycol were found. In this respect *Aerobacter* differed from similar fermentations by *Citrobacter freundii* where none of the latter products were found but some succinic acid was produced. Trimethyleneglycol production from glycerol cannot be used as a character to separate organisms of the intermediate colon types from *Aerobacter*.

NATURAL DISTEMPER IN GREY FOXES. *C. A. Evans and R. G. Green*, University of Minnesota.

During 1934 and 1935, an extensive outbreak of disease among wild grey foxes (*Urocyon cinereoargenteus*) occurred in southeastern Minnesota. Of thirteen foxes received, ten were studied for microscopic pathology, and the characteristic inclusion bodies of distemper were demonstrated in six. The other four were badly autolyzed. Cytoplasmic inclusion bodies were found in bile ducts, lymph node, adrenal, pancreatic ducts, and bladder.

Intranuclear inclusions, as is the rule in distemper, were less common than cytoplasmic, but were found in spleen, bile ducts, lymph node, adrenal pancreatic ducts, and salivary gland. All inclusion bodies appeared identical with those which characterize distemper in other animal species.

Paratyphoid (*Salmonella* sp.), a frequent secondary invader in cases of distemper in foxes on fox farms, was demonstrated in several of the grey foxes, including two with typical distemper inclusions. *Pasteurella pseudotuberculosis* was isolated from a fox in which both paratyphoid and distemper were also present.

Forty-five guinea pigs, six rabbits, and four quail were inoculated with material from the foxes, with essentially negative results. Four guinea pigs died of paratyphoid. Of four ferrets inoculated with tissue from three foxes, three died or were killed sick, but the presence of paratyphoid and of pseudotuberculosis makes these experiments of no more than confirmatory value in establishing the diagnosis of distemper.

Nonfatal Infections with Pasteurella tularensis in the Snowshoe Hare. *J. F. Bell and R. G. Green,* Department of Bacteriology and Immunology, University of Minnesota.

During an investigation of diseases of wild animals that has covered a period of eight successive years, tularemia has been studied in two species of rabbits, the snowshoe hare (*Lepus americanus-phaeonotus*) and the cottontail rabbit (*Sylvilagus floridanus-mearnsi*) on the Lake Alexander Area in Minnesota. Evidence has been adduced which indicates that the snowshoe hare, unlike the cottontail rabbit, is usually highly resistant to tularemia. The evidence is based on the epizootiology, pathology, immunology, and symptomatology of the disease in naturally infected snowshoe hares, and on characteristics of the organisms isolated from that species. In the period of the study, the incidence of infection in vectors of the disease became so great that cottontail rabbits on the Area were exterminated by tularemia; yet the snowshoe hares, which were more heavily infested by the vectors, did not sufer appreciable losses from this disease. In the snowshoe hares, tularemia usually was not acute, but was a symptomless infection characterized by chronic focal lesions. A high proportion of the hares trapped in winter possessed agglutinins for *Pasteurella tularensis,* an indication that they had recovered from infection with the organism. Strains of *P. tularensis* isolated from the snowshoe hare induced less acute infections in guinea pigs than did strains isolated from the susceptible cottontail rabbit.

Enzymic Variability of Aerobacter indologenes as a Function of Growth Conditions. *C. R. Brewer, M. N. Mickelson and C. H. Werkman,* Bacteriology Section, Iowa Agricultural Experiment Station, Ames.

Non-proliferating cell suspensions of *Aerobacter indologenes* grown on glucose in acid or alkaline buffer or on self-buffered citrate, possess enzyme systems which dissimilate other substrates to products resembling those of the "growth substrate." Pyruvic acid, a postulated intermediate compound, is fermented by "citrate" cells to give substantial yields of succinic acid with little 2,3-butylene glycol. Cell suspensions grown in acid glucose media dissimilate pyruvate to relatively high yields of the glycol and low quantities of succinic acid. These results conform to the normal dissimilation of glucose and citric acid.

A. indologenes cells grown on glucose in alkaline buffer or citrate, weakly attack glucose in acid buffer and form products normal to the dissimilation of alkaline glucose or citrate. Suspensions of cells grown in acid-buffered glucose dissimilate acid-buffered glucose to the normal products formed by growing cells. Alkaline glucose cells rapidly dissimilate glucose in alkaline buffer to the normal products of alkaline glucose fermentation by growing *Aerobacter.*

Suspensions of cells grown in alkaline-buffered glucose and in citrate are similar in enzymic activity. They are likewise similar in their growth metabolism. Acid glucose cells differ from both the above types in the activity of both growing and non-growing cells.

The Bacteriology of Perforation Peritonitis. *Cora R. Owen,* University of Minnesota, Minneapolis, Minnesota.

Guinea pigs were injected intraperitoneally with suspensions of the cecal contents of normal guinea pigs and

both the suspensions and the peritoneal cavities of the injected guinea pigs were cultured. Some of the suspensions were found to contain very few or no organisms of the coliform group and of the animals injected with this group of suspensions only 31% died, while 64% of the animals, injected with suspensions from which these organisms could be easily recovered, died. Members of this group of organisms were recovered from the peritoneal cavities of 84% of the guinea pigs which died of the injections and from only 16% of those which survived. It is concluded that the colon bacillus and related organisms are the most important pathogens in the intestines with regard to this condition.

Evidence for the Aerobic Decomposition of Lignin by Lake Bacteria. *Janice Stadler and Claude E. ZoBell**, University of Wisconsin, Madison.

Concentrations of purified lignin as high as 0.5 per cent are not toxic for bacteria from Lake Mendota and other Wisconsin lakes. The larger bacterial populations found in water enriched with lignin suggest that it is slowly utilized by aerobic bacteria. It was found that each of eleven samples of lignin, differing in either the process of preparation or source, was oxidized by bacteria as indicated by oxygen consumption in closed bottles of water. As much as 3.07 mgm. of oxygen was used by bacteria in the presence of 5.0 mgm. of lignin in 30 days at 28°C. The oxygen consumption data indicate that from 2.1 to 33.2 per cent of the lignin is oxidized during this period of incubation. Lignin abiogenically absorbs a little oxygen from water but the quantity is very small as compared to the amount which is utilized when the water is inoculated with an enrichment culture of lignoclastic bacteria. Estimating the lignin by the acid-permanganate method it was found that 10 to 20 per cent of certain lignin samples were decomposed by bacteria under aerobic conditions.

* On sabbatical leave from the Scripps Institution of Oceanography, La Jolla, California.

The Effect of Oxygen Tension on Oxygen Consumption by Bacteria in Lake Water. *Claude E. ZoBell* and Janice Stadler*, University of Wisconsin, Madison.

The oxygen tension of water from Lake Mendota was adjusted at different levels ranging from 0.66 to 7.99 mgm./l. and stored in glass-stoppered bottles. Dissolved oxygen was determined at the beginning of the experiment and after varying periods of incubation at 25°C. From the results it is estimated that during the first 24-hour period an average of 78×10^{-10} mgm. of oxygen was consumed per cell per hour, the amount being independent of the initial concentration of oxygen. Thereafter the rate of oxygen consumption dropped sharply probably due to the depletion of respirable organic matter but the rate was not influenced by the concentration of oxygen until the latter was exhausted. Similar experiments with resting cells in lake water enriched with organic matter confirmed the foregoing observations that the rate of respiration of certain lake bacteria is not a function of the oxygen tension of the water. This conclusion applies to *Serratia rubida* and *Flavobacterium flavus* in pure culture as well as to the mixed microflora found in Lake Mendota.

* On sabbatical leave from the Scripps Institution, University of California.

THE EFFECT OF SALICYLIC ALDEHYDE ON THE INFECTION OF WHEAT BY PYTHIUM ARRHENOMANES DERCHSLER, AND THE DESTRUCTION OF THE ALDEHYDE BY ACTINOMYCES ERYTHROPOLIS AND PENICILLIUM SP. *V. E. Graham and L. Greenberg*, University of Saskatchewan, Canada.

Salicylic aldehyde, when added to soil at the rate of 50 p.p.m., seems to predispose wheat roots to attack by parasitic strains of *Pythium arrhenomanes*.

Actinomyces erythropolis and a species of *Penicillium* have been found in soil from the healthy area of a field partially infected with Browning root rot. These organisms caused the disappearance of salicylic aldehyde in an artificial medium.

It is suggested that lack of activity on the part of such organisms in certain areas of a field may lead to an accumulation of salicylic aldehyde or products acting in a similar manner, and that this may be a predisposing factor in the appearance of Browning root rot caused by *Pythium arrhenomanes*.

When *Actinomyces erythropolis* and *Pythium arrhenomanes* are both added to sterile soil containing 50 p.p.m. of salicylic aldehyde under greenhouse conditions the harmful effect of the salicylic aldehyde is overcome.

CONCENTRATION OF POLIOMYELITIS VIRUS BY MEANS OF THE BEAMS CENTRIFUGE. *Weldon C. White and Paul F. Clark*, Univ. Wisconsin.

SOME EFFECTS OF STERILE INFLAMMATION ON EXPERIMENTAL POLIOMYELITIS. *A. F. Rasmussen, Jr. and Paul F. Clark*, Univ. Wisconsin.

A FERMENTATION CALORIMETER FOR THE STUDY OF HEAT EVOLUTION IN THE DECOMPOSITION OF PLANT MATERIALS. *R. E. Carlyle and A. G. Norman*, Department of Agronomy, Iowa State College.

There is little information about the phenomenon of heat evolution during the decomposition of plant materials and most measurements made up to the present have been confined to the determination of temperature rise. In an effort to make quantitative measurements an adiabatic fermentation calorimeter has been constructed. It consists of a vacuum flask capable of holding approximately 40 grams of material immersed in a bath the temperature of which is controlled by the temperature of the fermenting material inside. Two 2-junction thermopiles, one each in flask and bath, respectively, are connected directly to a galvanometer, the reflected beam from the mirror of which is focused on a photoelectric cell. Any deflection of the beam caused by an increase in temperature in the flask operates a relay switching on two knife heaters in the bath. The temperature in the flask is measured potentiometrically with a second thermocouple. The water equivalent of the calorimeter is determined by generating a known amount of heat electrically in a small resistance coil permanently in place in the calorimeter. Aeration is provided by passing air through a long copper coil in the bath and the air is saturated at that temperature by passage through a wet bead tower also immersed in the bath.

CENTRAL NEW YORK STATE BRANCH

THIRTY-SEVENTH SEMI-ANNUAL MEETING, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE, MAY 20, 1939

THE HYDROLYSIS OF DISODIUM PHENYL PHOSPHATE BY GRAM-NEGATIVE BACILLI. *Harold W. Leahy, Leslie A. Sandholzer and Marian R. Woodside*, University of Rochester, School of Medicine and Dentistry, Rochester, New York.

It has been demonstrated for the first time that a wide variety of Gram-negative bacilli (*Serratia*, *Pseudomonas*, *Escherichia*, *Aerobacter*, *Chromobacter*, *Proteus*, *Salmonella*, *Shigella*, *Eberthella* and *Alcaligenes*) are able to hydrolyze disodium phenyl phosphate. The dephosphorylating activity was present in the bacterial cells, but was absent in Berkefeld filtrates and supernates of centrifuged cultures.

The optimal hydrogen-ion concentration was determined by suspending, in 10 ml. of 0.005 M substrate solution buffered with either phthalate or veronal to pH values between 4.0 and 9.0, a weighed amount (from 2 to 5 mg.) of lyophilized cells which had been grown on plain agar. The phenol that resulted from hydrolysis of the phosphoric acid ester was determined by using Gibb's reagent (2,6-dibromoquinonechloroimide). All of the 23 organisms tested exhibited the presence of phosphatase. The optimal hydrogen-ion concentration, however, varied with the different genera and with different species in the same genus. The lowest optimum encountered was pH 5.8 and the highest pH 7.5. Under optimal conditions the maximal amount of phenol liberated was 1.12 mg. per mg. of dry cells in 24 hrs. at 37°C.

FERMENTATION OF CARBOHYDRATES BY STRAINS OF COMMERCIAL YEASTS. *E. A. Beavens*, N. Y. State Agricultural Experiment Station, Geneva.

ATTEMPTS TO APPLY SEROLOGICAL GROUPING TO THE NON-HEMOLYTIC STREPTOCOCCI. *J. M. Sherman, C. F. Niven, Jr., and Karl Smiley*, College of Agriculture, Cornell University, Ithaca.

STUDIES ON STAPHYLOCOCCI OF ANIMAL ORIGIN. *W. B. Bell*, Veterinary College, Cornell University, Ithaca.

THE NATURE OF VIRUSES. *George Packer Berry*, University of Rochester, Rochester.

CAN MICROÖRGANISMS BE USED TO INDICATE NUTRIENT DEFICIENCIES IN SOIL? *H. J. Conn*, New York State Agricultural Experiment Station, Geneva, New York.

The desirability of a quick test to indicate to what extent nutrient elements (particularly K and P) in soil are available to plants is recognized. Various microörganisms have been suggested for this purpose on the assumption that they have nutrient requirements similar to higher plants while their period of growth is so short that results can be studied in the laboratory. Although each method has its advocates, results have always been open to question. It is difficult to evaluate such results, because to get any basis of comparison a soil must be studied over a series of years with various crops. The writer has tried

unsuccessfully to use certain soil bacteria as test organisms, measuring their growth in soils by microscopic examination. It has been found that factors other than nutrient deficiencies (notably presence of colloids, and moisture level of the soil during the few weeks before the test is made) have had more effect on results than those that it had been desired to measure. These observations point to such great differences between the nutrient requirements of microörganisms and plants that it is doubtful if the former can be used to indicate deficiencies for the latter.

PROTECTIVE ANTIBODIES EFFECTIVE AGAINST TYPE I MENINGOCOCCAL INFECTION IN MICE. *Geoffrey Rake and Henry W. Scherp*, Squibb Institute for Medical Research, New Brunswick, N. J. and Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, New York.

A previous quantitative study of the precipitin reaction between a polysaccharide from Type I meningococcus and antimeningococcal horse sera indicated that monovalent sera contained only homologous type-specific antibody, whereas polyvalent therapeutic sera contain in addition large amounts of group-specific antibody. These findings have been correlated with the protective capacity of the sera against Type I meningococcal infection in mice. Complete absorption of sera with Type I polysaccharide removed from 90 to 99 per cent of protective antibodies. Partial absorption of polyvalent sera was carried out in such a fashion that all of the type-specific antibody, but only from 30 to 60 per cent of the group-specific antibody, was removed. The residual antibody, which constituted from 30 to 40 per cent of the total precipitable antibody of the serum, had very slight protective capacity. One serum was encountered, in which about one-fourth of the protective antibodies was absorbable by an "agar-hapten."

THE BACTERICIDAL EFFECT OF SULFAPYRIDINE IN VITRO ON THE GONOCOCCUS. *H. F. Wingate, R. Charles, and C. M. Carpenter, University of Rochester.*

The use of sulfapyridine for the treatment of gonococcal infections led us to investigate the bactericidal effects of the compound *in vitro*.

Forty strains of the gonococcus, isolated from various types of gonococcal infection, were used. Uniform suspensions in broth were prepared from the organisms grown for 24 hours on blood-glucose-ascitic fluid-agar slants. From each such suspension, 0.05 ml. was seeded into 1.5 ml. of blood-glucose-ascitic fluid broth containing enough sulfapyridine to yield a final concentration of 0.01 per cent (0.1 mg. per ml.). Control cultures without sulfapyridine were prepared in like manner. Serial subcultures were made on "chocolate" agar at 4-hour intervals for 48 hours, and incubated in 10 per cent CO_2 at 36°C. for 48 hours.

None of the strains was viable after 44 hours of exposure to sulfapyridine. One strain survived less than 4 hours, 2 failed to grow after 12 hours, 5 were non-viable after 16 hours, 10 after 20 hours, 9 after 24 hours, 9 after 28 hours, 2 after 32 hours, 1 after 36 hours, and 1 after 44 hours.

The 40 strains of the gonococcus were exposed in like manner to the same concentration of sulfanilamide, *i.e.*, 0.01 per cent. A comparison of the two compounds showed that both were equally effective in killing 6 of the

strains. Twelve strains were rendered non-viable in a shorter period of time by sulfapyridine, and 22 more quickly by sulfanilamide.

SCHLEIDEN AND SCHWANN AND THE MODERN CELL THEORY. *Leslie A. Sandholzer*, University of Rochester, Rochester.

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., MARCH 21, 1939

COLON GROUP BACTERIA AS THE CAUSE OF ACUTE FATAL DYSENTERY IN NEW BORN CALVES. *J. W. Dollahite*, Bureau of Animal Industry, U.S.D.A., Washington, D. C.

At a government-owned institutional dairy of about 250 cows, severe losses from dysentery in new born calves have occurred for a period of at least 12 years. During the first five and one-half months of 1938, 49 per cent of the new born calves died with acute dysentery before they were 5 days old. Cultures of *Escherichia communior* and *Escherichia acidilactici* were recovered from a large percentage of these calves that came to autopsy. A normal cow was hyper-immunized against these two cultures of bacteria and an immune serum prepared. Between June 15, 1938 and March 1, 1939, fifty-eight calves were treated with this serum. Of this total treated, four calves or 6.9 per cent died with dysentery. During the same period 65 calves were left untreated as controls. Fifteen control calves or 23.1 per cent died with acute dysentery. Colon-group bacteria were recovered from about 60 per cent of these calves that were autopsied.

COLONY AND ANTIGENIC VARIATION IN KLEBSIELLA PNEUMONIAE TYPES A, B, AND C. *W. A. Randall*, Georgetown University Medical School, Washington, D. C.

AN EPISODE IN THE HISTORY OF SMALLPOX VACCINATION IN NEW HAMPSHIRE. *M. C. Leikind*, Library of Congress, Washington, D. C.

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., APRIL 18, 1939

ORGANISMS INVALIDATING THE DIAGNOSIS OF GONORRHEA BY THE SMEAR METHOD. *George G. DeBord*, Health Department, District of Columbia.

Two undescribed Neisseria have been named. *Neisseria fulva*, from a case of conjunctivitis and vaginitis, has a waxy colony which does not adhere to the medium. The color is light tan, after mixing with a needle, a bright yellow. Acid is produced in glucose, fructose, maltose and sucrose. *Neisseria gigantea*, from a normal vagina, is a giant form with a waxy colony which can be moved over the medium with a needle. The colony is clear, becoming slightly opalescent with age. No sugars are fermented.

A new tribe, *Mimeae*, is proposed. The description follows: short rod, gram-negative, encapsulated, pleomorphic; growth on plain agar is abundant, white, glistening, smooth, viscid and the cells are almost wholly diplococcal in form, identical to the gonococcus in size and appearance; many cells retain the blue in Gram's stain in whole or in part; growth in broth is diffuse with a

viscid sediment with diplococci, rods and filaments present. Fermentation groups are (1) acid and gas from glucose, maltose, lactose with a few including sucrose, (2) acid only in glucose and maltose, (3) acid in glucose, (4) no sugars fermented. Motile and non-motile forms are found. Type species, *Mima polymorpha*, is a non-motile form from group four.

Approximately 30% of the total cases, normal and abnormal, showed organisms which might be mistaken for the gonococcus.

THE MORPHOLOGICAL, BIOCHEMICAL AND SEROLOGICAL PROPERTIES OF BACILLUS PASTEURIANUM AND ITS ABILITY TO FIX ATMOSPHERIC NITROGEN IN COMPARISON WITH OTHER ANAEROBIC BACILLI. *Howard L. Bodily*, Department of Bacteriology, University of Maryland. (This investigation represents studies carried out by the writer at the Department of Bacteriology and Public Health, University of Colorado Medical School.)

The generic term *Bacillus* was used in preference to *Clostridium* because, in the writer's opinion, selection of generic terms should be based on the most stable traits of bacteria, i.e., morphology and staining characteristics and not on physiological differences. Ten strains received as *Clostridium pasteurianum*, three strains received as *Clostridium beijerinckii*, and one strain received as *Bacillus amylobacter* A. M. et Bredemann were subjected to morphological, biochemical, and serological studies. The results indicated that only one strain, of which a description follows, could be identified as *Bacillus pasteurianum*. Young vegetative rods were motile by means of peritrichous flagella and were gram-positive. Older cells developed into clostridia which, except for one pole, were stained violet brown by iodine. Later they bore oval subterminal spores which when mature were retained within the mother cell in a "spore capsule." *B. pasteurianum* fermented glucose, galactose, mannose, levulose, sucrose, maltose, raffinose, inulin, glycerol, mannitol, sorbitol, and inosite, but failed to ferment xylose, arabinose, rhamnose, lactose, starch, salicin, dulcitol, gum arabic, and cellulose.

In two per cent glucose tryptone mineral medium, *B. pasteurianum* produced butyric acid, and small amounts of butyl and ethyl alcohols but no acetone or isopropyl alcohol. It failed to liquefy gelatin, to blacken deep iron brain medium, to digest casein, to reduce nitrates, and to produce acrolein, acetyl-methyl carbinol, and indol. Pathogenicity was negative for rabbits and guinea pigs. Cross-agglutination tests showed that *B. pasteurianum* was serologically unrelated to all the other strains studied. In addition to the above, 22 other strains of anaerobic bacilli were tested for their ability to fix nitrogen in a Winogradsky's nitrogen-free medium. The results showed that *B. pasteurianum*, although not exclusive in its ability to fix nitrogen, was most active. It fermented 100% of the glucose and fixed from 4.5 to 4.6 mg. of nitrogen in 100 ml. of medium.

THE VIRUS OF PSITTACOSIS

I. Propagation and Developmental Cycle in the Egg Membrane, Purification and Concentration[1]

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INTRODUCTION

Psittacosis is a latent or manifest infection of psittacine birds. The disease is transmissible to man and is characterized by high fever simulating a typhoidal state and associated with symptoms of an atypical pneumonia. The infection is occasionally transmitted from man to man. However, when parrots or parrakeets are kept as pets, household epidemics have frequently been observed.

The term psittacosis is derived from the Greek ψιττακός—parrot, and was first applied by Morange in 1895. Parrot fever as a technical term does not apply to a disease of parrots so much as to a characteristic disease of man, which is contracted by contact with psittacine birds. The unfortunate impression conveyed by the term "parrot fever" has led to the belief that the parrot is the sole offender, whereas the shell parrakeet plays a significant rôle, especially in California, as pointed out by Meyer and Eddie (1933a, 1933b, 1934) and by Meyer (1935). Canaries, finches and other birds may be carriers of the virus and cause human infections (Meyer (1938), Pfaffenberg (1936)). A bird of the petrel family has been shown to be responsible for outbreaks of human psittacosis (Haagen and Mauer (1938a)).

[1] Portion of a thesis submitted by the senior author in partial satisfaction of the requirements for the degree of Doctor of Philosophy, University of California.

[2] Edith Claypole Memorial Research Fellow in Pathology, 1936–1938.

The review by Elkeles and Barros (1931) gives a complete history of the early knowledge of the disease and an excellent summary of the pandemic of 1929–1930. More recently Pfaffenberg (1936) has made a splendid review of the current status of the disease and the progress of research studies, while Meyer (1935) has summarized pertinent data concerning the epidemiology of human infections in the United States. The Ministry of Health Report (1930) reviews the 1929–1930 pandemic, with special emphasis on English experience. German observations have been analyzed by Haagen and Krückeberg (1937) and Haagen and Mauer (1938b).

Concerning the etiologic agent of the disease, the work completed prior to 1929 is principally of historic interest. In 1893 Nocard isolated a gram-negative bacillus from the bone marrow of parrots dead of psittacosis. He considered this organism to be the causal agent and named it in consequence *Bacillus psittacosis*. This organism was not found consistently in either human beings or birds and was recognized by Bainbridge (1912), Perry (1920) and subsequent workers to be identical with *Salmonella aertrycke*.

The inconstant bacteriological findings prompted investigators to search for a filterable virus during the pandemic of 1929–1930, with the result that early in 1930 the German workers under Levinthal, the English group under Coles and Bedson and the United States workers under Lillie independently established the virus nature of the disease. Almost simultaneously Levinthal (1930a), Coles (1930) and Lillie (1930) described small coccoid or diplococcoid bodies in infectious material, with a size estimated by measurement of stained preparations and photomicrographs as ranging from 0.20 to 0.45μ. Furthermore Coles (1930) passed material through Seitz filters and found the coccoid bodies in the infectious filtrates. Levinthal (1930b) likewise found filtrates of Berkefeld V candles to be infectious. Lillie (1930), noting the resemblance of the bodies observed to the rickettsia group, proposed the name "*Rickettsia psittaci*" for the "minute gram-negative intracellular coccoid and bipolar bacilliform bodies of about 0.2μ to 0.3μ diameter, found in

reticulo-endothelial cells, mesothelial cells and large mononuclear cells of the parrot (probably Amazon sp.) and in large mononuclear cells in man, associated with, but without established relationship to, psittacosis." As pointed out by Meyer (1935) there is no evidence that psittacosis is an insect-borne disease, and the name "*Rickettsia psittaci*" is not appropriate, despite the intracellular location and poor staining properties of the bodies. Levinthal (1930a) proposed the name "*Microbacterium multiforme psittacosis*" for the cellular inclusions, but this terminology has not been accepted by other workers.

Apart from the occasional elongated forms, the resemblance to the Paschen bodies of vaccinia and variola is quite marked, and, following the traditional custom of naming inclusion bodies after the discoverer, Meyer and Eddie (1933b) proposed the name "Levinthal-Coles-Lillie bodies" which was shortened to "L.C.L. bodies." This designation has found general acceptance in both the American and foreign literature, and will be used throughout this report. The virus nature of the L.C.L. bodies in the generally accepted meaning of the term (requiring living tissue for multiplication) has been further confirmed by the recent work of Haagen and Crodel (1936) and of MacCallum (1936) in detailed studies showing the necessity for living cells to obtain multiplication of the virus.

The susceptibility of the white mouse to intraperitoneal injections of psittacosis virus was established by Krumwiede, McGrath and Oldenbusch (1930), and the use of this animal has proved invaluable in research studies. The high contagiousness of the disease is well known, especially when infected birds are being handled and cared for. Of the eleven accidental infections contracted by the personnel of the National Institute of Health, McCoy (1930) has reported that eight individuals had no exposure to infected birds, two were exposed, and one case was doubtful. Due to the danger in handling active virus, the range of experimental studies was limited and certain desirable information was not obtainable for this reason. Since laboratory infections are relatively frequent, all known precautions were taken throughout the present study and certain omissions and

incomplete data may be ascribed to the danger of carrying out doubtful procedures.

PROPAGATION OF THE VIRUS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING EGG

Review

Since the early reports of Goodpasture and his co-workers (Woodruff and Goodpasture (1931), Goodpasture, Woodruff and Buddingh (1931), Goodpasture, Woodruff and Buddingh (1932)) the chorio-allantoic membrane of the developing chick has been used as a culture medium for a number of viruses and rickettsiae. Among the agents reported propagated on this medium are vaccinia (Goodpasture, Woodruff and Buddingh (1931)), fowlpox (Woodruff and Goodpasture (1931)), varicella (Nauck and Paschen (1932)), herpes (Dawson (1933)), infectious laryngotracheitis (Burnet (1934)), vesicular stomatitis (Burnet and Galloway (1934)), Rift Valley fever (Saddington (1934)), influenza (Smith (1935)), alastrim (Torres and Teixera (1935)), equine encephalomyelitis (Higbie and Howitt (1935)), psittacosis (Burnet and Rountree (1935), Fortner and Pfaffenberg (1935)), louping ill (Burnet (1936a)), lymphocytic choriomeningitis (Bengtson and Wooley (1936)), sandfly fever and dengue fever Shortt, Rao and Swaminath (1936)), ectromelia infectiosa (Paschen (1936)), common cold (Kneeland, Mills and Dochez (1936)), St. Louis encephalitis (Harrison and Moore (1936)), variola (Lazarus, Eddie and Meyer (1937a)), rabies (Kligler and Bernkopf (1938)), typhus fever (Zia (1934)) and Rocky Mountain spotted fever (Bengtson and Dyer (1935)). The rapidly growing list of viruses and rickettsiae propagated on this medium in a comparatively short time attests both to the efficiency of the method and to its general simplicity.

In addition to the successful propagation of the virus of psittacosis on the chorio-allantoic membrane (Burnet and Rountree (1935), Fortner and Pfaffenberg (1935)), the literature shows reports of other methods which have been successfully applied to the study of the virus. Levinthal (1935) described multiplication of the virus in the endothelial and epithelial cells

of minced chicken embryo incubated with 5 ml. of Tyrode solution in Carrel flasks, while Haagen and Crodel (1936) report 30 culture passages with undiminished virulence for mice by using single drop cultures and Maitland mass culture technique. Bland and Canti (1935) used the hanging drop method for photographic study of the developing forms of the virus, using darkfield technique, while MacCallum (1936) showed the need for living cells to produce multiplication of the virus.

Technique

Two methods of propagation of psittacosis virus in eggs have been used in this study and have been found to be uniformly satisfactory. The cover-slip method has been used when it has been necessary to watch the progress of the infection and to note the time of death of the embryo. The shell-flap method has been used for routine passages and where large quantities of the virus were desired. Goodpasture and Buddingh (1935) give a complete description of the technique of inoculation.

For the purposes of this study, 9 or 10 day embryos were found to be most satisfactory. After inoculation the eggs were placed in an incubator kept at a temperature of 35.5 to 36.5°C., it having been observed that the number of dead embryos was much reduced by lowering the temperature of the incubator containing the inoculated eggs. Further reference will be made to the reasons for this fact. Humidity was kept relatively high by keeping pans of fresh water in the incubator, but the eggs could not be turned after inoculation by these methods.

After incubation of the infected eggs for the proper period, the membranes were removed aseptically. The cover-slip or shell-flap was lifted off and the shell carefully broken with sterile forceps until the membrane was exposed. With experience this procedure could be carried out on the open laboratory bench without any contamination of the tissue. The membrane was cut out with sterile iridectomy scissors and placed in a Petri dish. Portions were then cut from the infected area for use in staining or transfer.

Routine staining was carried out using the original method of

Castaneda (1930) and the standard Giemsa method. The Castaneda stain was more satisfactory for checking infected tissue before transfer, since the presence of L.C.L. bodies and the absence of bacteria could be rapidly verified and the entire staining procedure was completed in less than 5 minutes. The Giemsa stain gave more clean-cut microscopic pictures and was more valuable for photomicrographic purposes.

The victoria blue stain of Herzberg (1936) has given excellent results, although no counterstain could be used and it was sometimes difficult to identify the virus elements inside the cells. Victoria blue "Bayer" was made up in a saturated aqueous solution (3 per cent) and allowed to settle overnight. Immediately before using, 5 per cent saturated citric acid was added to the stain. The smears were air-dried for 24 hours and then covered with the mixture of dye and citric acid. After 2½ to 3 minutes, the slide was rinsed in tap water and blotted. Examination was made under the oil immersion lens without further staining. The elementary bodies appeared a dark blue against a lighter blue background of tissue cells.

The Macchiavello stain suggested by Zinsser (1937) yielded valuable results. Smears were fixed with moderate heat and stained for 3 minutes with a solution containing 0.25 per cent basic fuchsin in phosphate buffer or distilled water at pH 7.4. The slides were then rapidly washed with 0.5 per cent citric acid in distilled water. Counterstaining was rapidly done with 1 per cent methylene blue. L.C.L. bodies appeared bright red against a pale blue background.

Virus strains used

Three strains of psittacosis virus were used in this study. The "Australian OH" strain was isolated in September 1933 from shell parrakeets received from Australia and has had no known connection with human cases. The "Rheinen" strain of the virus was isolated from the sputum of a human case of the disease contracted by contact with infected shell parrakeets in September 1934, while the "Maine" strain was isolated from shell parrakeets received from Portland, Maine in February 1934 and

has had no known connection with any human case. These three strains were passed routinely through susceptible white mice and occasional birds until they were inoculated into developing eggs.

Experimental procedure

In September 1935, using 1 drop of a 10 per cent mouse spleen emulsion in broth, the Australian OH strain was successfully propagated and transferred in eggs. Due to lack of training in the technique and to incubation at too high a temperature, approximately 50 per cent of the early passages resulted in death of the embryo. As the technique improved and the necessity for lower incubation temperatures was recognized, this percentage decreased until it has become negligible. All membranes were checked with the Castaneda stain before passing and no membranes from eggs containing dead embryos were used when it was possible to obtain freshly sacrificed material. Routine bacteriological cultures on blood agar, Avery's broth and brain broth were consistently negative throughout this study.

Early passages were accomplished by transferring a piece of infected membrane 1 mm. in diameter to a newly exposed membrane. The first 14 passages resulted in death of the embryo in from 5 to 6 days, with positive findings in membrane, spleen and liver impression smears with the Castaneda and Giemsa stains. After the 14th passage, death began to occur earlier and was stabilized at from 4 to 5 days by the time the 22nd passage had been reached. One ml. of ground 10-per-cent membrane emulsion in broth from the 4th and 5th passages injected intraperitoneally into white mice killed the animals in from 3 to 4 days with typical psittacosis lesions.

The liver was removed from an embryo in the 3rd passage and a 1 mm. piece of the tissue was transferred to a newly exposed membrane. Typical positive membrane results were obtained and the liver to membrane passage was continued up to the 20th passage. Precautions were taken to assure removal of the liver without fouling the tissue with the infected membrane. Grossly and microscopically, the membranes inoculated

in this manner were indistinguishable from those inoculated with infected membrane material.

The Rheinen and Maine strains were established in egg membranes in a similar manner. Infection with the Rheinen strain resulted in death of the embryo in 3 to 5 days after the virus became "fixed" by 10 preliminary passages. The Maine strain was able consistently to kill the embryo in 3 to 4 days after inoculation when 15 preliminary passages had been made. Liver and spleen were found to contain the virus in all cases, and successful transfers were made using fragments of these organs as inocula.

Several attempts to produce membrane infection with rather weak mouse spleen suspensions failed. Attempts to use membrane material stored in the ice box for 18, 20 and 25 days failed to produce any positive results in the chorio-allantoic membrane. While the egg membranes in both cases were bacteriologically sterile, L.C.L. bodies were either absent or found in very small numbers.

The Maine strain of psittacosis virus was used in the following studies. The virus has been passed from egg to egg for the past 38 months and at the time of writing has undergone over 425 consecutive bacteria-free passages without any intervening animal passage. The virus kills the embryo regularly in from 72 to 96 hours and the infectiousness for susceptible white mice has remained unaltered, as will be shown in later sections. Passage is made 3 times weekly, once after 72 hours incubation and twice after 48 hours. Nine to 10 day eggs are used exclusively and a minimum of 6 eggs is used at each passage. No bacterial contaminations have been encountered in the past 32 months. In addition to the experimental studies below, virus has been supplied to other investigators for use in animal immunization and complement fixation studies.

Observations during egg propagation

Using a tissue inoculum, the first signs of infection of the chorio-allantoic membrane appeared about 40 hours after inoculation. The area in the immediate vicinity of the primary infection became grayish and edematous, and the lesion spread

rapidly throughout the membrane, until at about 70 hours the entire exposed surface was thickened and gray, but without sharply defined lesions. This was in marked contrast to the observations of Burnet and Rountree (1935) who described discrete focal lesions about 0.25 to 1.0 mm. in diameter. In our experience with over 4,600 inoculated eggs, discrete foci were seldom observed, regardless of the form or dilution of the inoculum. The membranes were quite thickened by edema and in 72 hours might measure as much as 2 mm. in cross section. The general opacity noted in these infected membranes was caused by the cellular infiltration between the ectoderm and entoderm. If the infection was allowed to progress, the embryo succumbed between the 3rd and 4th day and the edema rapidly disappeared from the tissue. The original inoculum became necrotic and was occasionally surrounded by a small amount of dried extravasated blood. Occasionally membranes would be extremely dry and free from edema. This was probably due to physical conditions in the shell, allowing moisture to evaporate from the tissue surface.

Using a liquid inoculum of the Maine strain, the same gross picture resulted in the membrane, with the time of death varying in accordance with the dilution employed. If 0.1 ml. of undiluted saline-virus suspension, prepared in a manner to be described later, was used, the resulting membrane infection was not grossly distinguishable from that following a tissue inoculum, except of course the absence of the tissue fragment used as the inoculum. The time of embryo death was not altered from the above when the undiluted material was used. A 1 in 100 dilution of the virus-saline suspension resulted in death of the embryo in 5 to 6 days, while a 1 in 1,000 dilution caused death in 6 to 8 days. The eggs inoculated with more dilute material usually hatched and because of the danger of laboratory infection, these chicks were immediately destroyed and the shells and dried discarded membranes were autoclaved.

The membranes resulting from these diluted inoculations were similar to those from undiluted material, except that the surface drying due to prolonged incubation was more pronounced. In

no case was it possible to produce a proved infection without embryo death. Burnet and Rountree (1935) in their studies used an admittedly weak strain of bird virus, and in their discussion state that "it would be of great interest to know whether more virulent strains derived from or associated with human cases showed a capacity to overcome this defense mechanism of the egg."

Regarding the embryo proper, no skin lesions have ever been observed. The organs underwent some changes, as would be expected from the proved presence of virus in the viscera. The spleen was enlarged and the liver occasionally showed areas of fatty necrosis, usually confined to the median margins of the lobes. The normal size of the organs was exceedingly difficult to standardize, since the embryos underwent their most rapid period of growth during the age at which they were used for this type of work. In addition, two embryos of the same incubation age may show a tremendous variation in size, and, thus, a statement that any organ had become enlarged could only be made when the tissue showed a hypertrophy out of all proportion to the other organs.

A previously undescribed finding was the production of water blebs in the amniotic sac of heavily infected eggs. These blebs were round and raised and attained a size of 5 mm. in diameter. They were not always present and probably represented the reaction of the tissue to an overwhelming infection, with an accompanying increase in temperature. That they are entirely non-specific for psittacosis was shown by the fact that they have also been observed in eggs heavily infected with vaccinia and variola viruses and in uninoculated eggs incubated at higher temperatures than normal. From the fact that these blebs could be produced in normal eggs by raising the incubation temperature to around 41°C. for about 24 hours, it appeared reasonable to assume that they were the result of temperatures above normal.

These facts supplied a logical explanation for the observation that infected eggs showed a much lower mortality rate when incubated at temperatures around 35.5°C. This applied to vaccinia and variola viruses as well as to psittacosis and has been

observed by other workers in this field without any attempt to supply an explanation (Burnet (1936b)). In the face of the observations made, it appears reasonable to state that the infected embryo maintains a higher body temperature than is normal, and that by keeping the surrounding environment at a lower temperature, the egg may lose heat rapidly enough to delay death of the embryo. A careful search of the clear fluid inside these blebs failed to reveal any elementary bodies in any type of infection, and it must be concluded that they were the result of an overwhelming infection with an accompanying rise in temperature.

Microscopic observations

Microscopically, the membrane yielded valuable information. The first positive findings in impression smears were observed about 40 hours after infection. Castaneda stains of the membrane showed both ectodermal and leucocytic cells to contain early enlarged virus bodies, approximately 0.8 μ in diameter. These bodies were frequently elongated, and apparently divided by simple fission to produce the clusters of 4 to 8 intracellular L.C.L. bodies also found in these early preparations. A similar mechanism, elongation followed by binary fission, has been demonstrated for ectromelia elementary bodies by Barnard and Elford (1931).

As the infection progressed, the small clusters of L.C.L. bodies underwent rapid increase in size, so that by the time the peak of the invasion was reached in 72 hours, the ectodermal cells were tremendously enlarged, and frequently contained masses 20 μ in diameter. Around the 3rd day of the infection, these enlarged cells burst and liberated great numbers of free L.C.L. bodies. The smears of the 72-hour membranes showed many ectodermal cells containing these huge "colonies" as well as numerous free bodies broken from the infected cells by pressure and by mechanical trauma in preparing the smears. The 72-hour membranes also showed some few early forms, showing that the first stages may have caused a second invasion of previously uninvolved cells. In membranes from recently dead

embryos the L.C.L. bodies were present in the free state in tremendous numbers, probably as the result of tissue autolysis after the death of the embryo. Several thousand free bodies per oil immersion field have often been observed in Castaneda-stained smears of such membranes.

An interesting observation in the impression smears of infected membranes has been the apparently increased size of the individual elementary body. Microscopic examination has shown the chicken membrane L.C.L. bodies to be approximately one-third larger than the elementary bodies found in mouse material when stained by the same method. When virus suspensions prepared from egg membranes were inoculated into white mice, the resulting elementary bodies in the mouse were smaller than those in the original material, but occasional larger forms were observed with regularity. This disparity in the size of the virus bodies from different hosts may possibly be accounted for on the basis of the egg membrane being a more favorable medium, as evidenced by the great number of L.C.L. bodies found, or it may have been caused by the reaction of the stain with the protein of the host. In view of the difficulty encountered in filtering egg membrane virus, as reported below, there was some basis for believing that an actual increase in the size of the virus body had occurred. However, ultrafiltration studies showed the particles from different hosts to be identical, or nearly so, in size and it must be concluded that the observed microscopic increase was due to the protein of the host forming a film which reacted with the dye to give an impression of a relative increase in size.

Impression preparations of the liver and spleen of 72-hour infected embryos have shown definite inclusion and elementary bodies, but in no such quantities as in membrane smears. The small amount of virus demonstrable in these organs, together with the large amount of fat and extraneous material, precluded the possibility of using these tissues as a source of large quantities of virus. The organ involvement of the chick embryo is in contrast to the findings of Burnet and Rountree (1935) who reported an absence of the virus in the spleens of embryos with gross

membrane lesions, using a strain of psittacosis only moderately virulent for mice.

The amniotic fluid was consistently negative for the presence of virus, both by microscopic examination and by mouse inoculation. With the presence of the virus in the organs of the embryo proved, the only means of transport from the membrane to the embryo without contamination of the amniotic fluid was by way of the blood stream. Microscopic examination of blood smears prepared from the heart blood and from the umbilical vessel blood showed definite evidence of minute quantities of virus.

An interesting finding also observed by Burnet and Rountree (1935) was the presence of large red-staining bodies in Castaneda preparations of 72-hour membranes. These forms may reach a size of 2 μ in diameter, are invariably extracellular and tend to appear in groups up to 10 in number. Burnet and Rountree (1935) have called these clusters early forms of virus, an interpretation not necessarily proved as yet. In view of the extracellular nature of these forms and their presence in Castaneda-stained membranes, it is believed that these clumps of indeterminate nature may represent artefacts resulting from coagulation of protein material from the formalin used in this stain. These bodies have not been described in sectioned material, nor have they ever been observed in sediments of centrifuged material. There are three possible explanations for the presence of these forms: (1) They may be portions of the encapsulating membrane surrounding virus "colonies" as described by Bland and Canti (1935). (2) They may be artefacts resulting from the action of formalin on the nuclear material present in such large quantities in an overwhelming infection. (3) They may be stages in the actual development of the virus. Due to the absence of these forms from sediments containing large numbers of L.C.L. bodies, it is felt that they were more likely to be the result of the staining procedures than a stage in the infection. It is of interest to observe the gross similarity of these forms to the new group of saprophytic filterable organisms recently described and pictured by Laidlaw and Elford (1936).

Discussion

The findings with egg membrane propagation fully confirmed the statements of Bedson and Western (1930a, 1930b) and Meyer (1935) that the number of L.C.L. bodies runs parallel with the virulence. Attempts to cultivate the virus on the membrane failed with weak suspensions of mouse material, while the rapid loss of virulence of stored membrane material was noteworthy. This bears out the impression of Burnet (1936b), who stated that the mouse was a more sensitive medium for the detection of the virus than the egg membrane. Highly virulent mouse material resulted in an overwhelming infection of the membrane with the production of great numbers of elementary bodies, which has made possible over 425 uninterrupted passages of the membrane virus. Likewise, when large numbers of elementary bodies were present, the embryo succumbed, which was not the case if only a few L.C.L. bodies were found. Accompanying the production of numerous elementary bodies has been an unaltered infectiousness for the white mouse after 38 months of consecutive egg membrane passage. Obviously a heavy original inoculum was necessary but once multiplication commenced, the embryonic cells supplied such a splendid medium for continued reproduction that highly virulent material was produced constantly. From these findings and from evidence to be presented in later sections, *it appears obvious that the elementary body is the actual virus particle, a view that is becoming more and more generally accepted as evidence accumulates.*

The question of a developmental cycle for psittacosis virus has been the subject of much interesting work. Bedson and Bland (1932) thoroughly studied the development of the cellular inclusions and favored for a time the hypothesis that the virus was not a bacterium but possessed protozoal affinities with a developmental cycle of ameboid forms, or plasmodium, morula, division and subdivision of morula elements and finally elementary bodies. This hypothesis was withdrawn later (Bedson (1933), Bedson and Bland (1934)) and the authors now agree with the opinion that this virus is a microorganism with bacterial affinities (Levinthal (1935)). Two forms of intracellular

development have been postulated by Levinthal (1935), depending on whether or not the invaded cell was normal. Bland and Canti (1935) in a splendid photomicrographic study using chick embryo tissue cultures showed the existence of a developmental cycle and the presence of a membrane encapsulating the colonies of elementary bodies. Burnet and Rountree (1935) discussed the development of the virus in egg membranes, and on the basis of the results herein reported, the major conclusions of the Australian workers regarding a developmental cycle are fully confirmed.

Briefly, on the basis of Castaneda and Giemsa stained smears, the observations of the developmental cycle of this virus may be summarized as follows: (1) The earliest forms found in ectodermal cells were larger than the mature elementary bodies and represent the simple increase in size of the single L.C.L. body after entering the cell. The enlarged size probably represented the influence of lack of pressure from other inclusions and the result of more intimate contact with the cytoplasm. These larger forms may multiply in the protoplasm and diffuse throughout the cell, thus giving rise to several colonies in one cell. If smears were made at this stage, the larger forms (about 1 μ) were occasionally found free as a result of mechanical trauma or actual liberation from the cell. This form stained blue with the Castaneda stain. (2) The colonies were formed by simple multiplication of the original infecting particle, probably by binary fission. At this stage small clumps of discrete elementary bodies, blue staining with Castaneda's method, were observed inside the cytoplasm and small free clumps were likewise seen in impression smears. Division by simple binary fission was further verified by the presence of many intra- and extracellular diplococcoid forms in this stage. These forms stain blue by the Castaneda stain. (3) As the colonies increased in size, the encapsulating membrane developed, whether from the virus particles or from the cellular protoplasm could not be determined. If more than one group was developing in a single cell, these colonies may fuse to form the very large clumps of elementary bodies found in the ectodermal cells. The early clumps of virus surrounded by the capsular membrane

may account for some of the red-staining bodies seen with the Castaneda method. (4) The membrane ruptured as the elementary bodies increased and the cell weakened. Large numbers of free bodies were released. It was doubtful if these free forms could re-enter susceptible cells in egg membranes, since the entire cycle took approximately 72 hours and the embryo succumbed after that time, a fact of considerable interest and significance. What early forms there were in 72-hour preparations may be accounted for by the release of large forms from stage (1). The appearance of the so-called "morula" forms could not be confirmed, but these may result in mouse material as described by Bedson and Bland (1932) due to differences in protoplasmic density or other physical causes.

SUMMARY

1. The virus of psittacosis has been propagated on the chorio-allantoic membrane of the developing egg for a period of 38 months without any loss of infectiousness for mice. Over 425 consecutive bacteria-free passages have yielded material for a wide variety of studies.

2. Psittacosis virus has been consistently recovered from the spleen and liver of the embryos.

3. The virus has become "fixed" for the host and causes death of the embryo between the 3rd and 4th days, with a probable increase in the temperature of the embryo.

4. A simple developmental cycle has been postulated and demonstrated on the basis of stained smears from infected membranes.

PREPARATION OF VIRUS SUSPENSIONS

Review

The literature contains little reference to the preparation of suspensions of psittacosis virus. The work on the complement fixation reaction (Bedson (1935), Levinthal (1935)) refers only to the use of dilute suspensions of infected organs of mice. The egg membrane studies of Burnet and Rountree (1935) make no reference to uses of the virus outside of microscopic examination.

Since this type of material supplied a rich source of large amounts of virus, it was considered of value to prepare suspensions of virus that could be used for more specific investigations. The general methods followed have been outlined by Craigie (1932) in reference to vaccinia and by Rivers and Ward (1937) in reference to infectious myxomatosis. Further mention will be made of these applications as the methods are outlined.

Methods of preparation of crude suspensions

The presence of large numbers of L.C.L. bodies in impression smears of infected egg membranes gave promise of large yields of relatively pure virus. The mouse spleen and liver, on the other hand, contained relatively small amounts of virus and large amounts of fat and extraneous tissue material. It was soon observed that the egg membranes could be washed in buffered saline or buffered water and that the washings contained large amounts of virus when titrated by intraperitoneal inoculation into susceptible white mice of a common stock.

The buffered water referred to in this and future sections was prepared according to McIlvaine's standards using mixtures of 0.2 M Na_2HPO_4 and 0.1 M citric acid (Clark (1925)). These mixtures were diluted 1 in 50 by the addition of distilled water. The buffered saline contained 0.85 per cent NaCl in addition to the above. The pH was checked by phenol red after the solutions were autoclaved.

In preparing suspensions of L.C.L. bodies, an arbitrary standard of 2 ml. of diluent per membrane was set up. The infected area of different membranes varied considerably in size, but large numbers of membranes were used, and the arbitrary standard yielded uniformly satisfactory results.

In general, the procedure followed was to shake the infected membranes with the diluent in a mechanical shaker in a 50 ml. Erlenmeyer flask for from 20 to 30 minutes. The mixture was then centrifuged in the ordinary laboratory centrifuge for 15 to 30 minutes at 3,000 revolutions per minute in order to throw down gross tissue particles. The resulting serum-colored supernatant fluid was removed, the sediment resuspended, shaken and

centrifuged once more. The supernatant was removed and pooled with the first. The pooled suspension was centrifuged once more for 20 minutes and the supernatant fluid was used as a basic suspension for further work.

Occasionally, the membranes were minced before shaking, with little apparent effect. If the shaking was done in the presence of glass beads, large amounts of soluble protein were present in the final crude suspensions and little increase in virus content was evident. For this reason, the membranes were handled gently,

TABLE 1

Maintenance of potency of crude suspensions after 309 passages

Date	10/9/35		6/11/36		3/1/38	
Egg passage number	8		57		317	
Strain	Maine		Maine		Maine	
Straight	K3+	D3+	K2+	D3+	D2+	D3+
10^{-1}	D5+	K5+				
10^{-2}	K6+	K6+	D6+	D6+	D4+	D5+
10^{-3}	D6+	K6+				
10^{-4}	K8+	K8+	D6+	D6+	D8+	D9+
10^{-5}	K8+	D8+	D7+	D7+	D7+	D9+
10^{-6}	K8+	D8?	D9+	D9+	D10+	D15+
10^{-7}	D11+	D12+	D8+	D13+	D10+	D13+
10^{-8}	D12+	S61+	D12+	D15+	D15+	D19+
10^{-9}	S48−	S48−	D11+	S49−	D21+	S60−

S = mouse sacrificed, not ill, D = mouse dead, K = mouse killed *in extremis*, + = typical findings for psittacosis, − = negative for psittacosis, ? = findings doubtful. Figures indicate days after inoculation.

in order that the final supernatant fluid would contain as little foreign material as possible.

Results

The crude suspensions were remarkably uniform if used shortly after preparation. Table 1 shows the titration of crude suspensions in white mice and illustrates the maintenance of potency after numerous transfers in egg membranes.

All inoculations were done within 3 hours after the completion of the preparation of the suspension. The amount injected was

invariably 0.5 ml. and intraperitoneal inoculation was the method of choice.

Table 1 illustrates not only the maintenance of virulence for white mice after numerous egg passages but also demonstrates another interesting finding, namely the tendency for death to occur in 3 days after inoculation, or in multiples of 3. This observation has been repeatedly made, and the phenomenon was probably the result of the release of large numbers of elementary bodies at the end of the 72-hour cycle already described.

All autopsies were done as soon as possible after death. The appearance of the internal organs was used as the criterion for the presence or absence of infection. The changes in the viscera are well described by Levinthal (1935). In some cases, the presence of the virus was verified by staining impression smears of the peritoneal exudate by the Castaneda or Giemsa methods.

This type of crude suspension rapidly lost its infectiousness for white mice after preparation. Storage in the ice chest at +4°C. was of little value in maintaining the potency of the material, and after 24 hours a definite decrease in infectiousness was apparent. After 5 days, little active virus was present and the suspensions generally were non-infectious in 10 days. This fact made it necessary to plan experiments so that the virus could be used shortly after preparation was completed. All suspensions were kept in the ice chest between the time of preparation and the time of actual inoculation.

Crude suspensions of the type described were used for the filtration, centrifugalization and purification studies to be discussed in later sections. In addition, these suspensions were used as an antigen for the study of the complement-fixing and neutralizing antibodies in studies by other workers.

Preparation of purified suspensions

The presence of large amounts of soluble protein in the crude virus suspensions made them of doubtful value for accurate serological work. It was accordingly decided to attempt further purification of this type of material.

The studies of Ledingham (1931), Craigie (1932), and Craigie

and Wishart (1934a) followed by the work of Parker and Rivers (1935) on the virus of vaccinia gave definite proof that the elementary bodies of that disease could be concentrated and washed by means of differential and fractional centrifugalization. Using these methods, the psittacosis virus crude suspensions were subjected to further purification.

The preparation of the crude suspensions was as already described, with one additional step. After removal from the egg, the infected membranes were rinsed in two changes of buffered saline, pH 7.2 to 7.6. Following this, they were then briefly rinsed in petroleum ether to remove part of the lipoid substances and were then again rinsed in buffered saline. The membranes were then used for the preparation of crude suspensions as already described. This treatment removed the mucilaginous material adherent to the surface as well as blood which had oozed from the cut vessels of the membranes. Table 2 shows the non-toxicity of the petroleum ether for the psittacosis virus.

TABLE 2

Demonstrating lack of toxicity of petroleum ether

DILUTION OF VIRUS	BEFORE WASHING WITH PETROLEUM ETHER		AFTER WASHING WITH PETROLEUM ETHER	
Straight	D3+	D3+	D3+	D4+
10^{-2}	D6+	D7+	D6+	D6+

D = dead, + = positive findings for psittacosis. Figures represent days after inoculation.

The virus used for the experiment summarized in table 2 was from the 294th egg membrane passage. Half the membranes were treated according to the standard method for the preparation of crude suspensions. The remainder were treated in the same manner after washing with petroleum ether as described. The titrations in table 2 show that the brief exposure to the petroleum ether was not sufficient to decrease the infectiousness of the virus.

The crude suspensions prepared from the washed membranes were then placed in flat Pyrex tubes with an inside diameter of 4 mm. and a capacity of 4 ml. and run in a Swedish angle centrifuge for 1 hour at 4,500 R.P.M. The use and advantages of

this instrument will be discussed in a later section. The supernatant fluid was poured off and saved for titration of precipitinogens. The angle sediment, which contained practically all of the elementary bodies as well as considerable amounts of tissue materials, was taken up in 5 to 10 ml. of buffered water, pH 8.2. To this suspension was added 0.5 ml. of the Seitz filtrate of a 1 per cent solution of Fairchild commercial trypsin. This trypsin had previously been freed from lipoids by successive extraction in the Soxhlet apparatus with ethyl ether and petroleum ether, for 6 hours each. The suspending fluid was kept at a relatively high pH in order that the trypsin might work in its optimum range. Fat-extracted trypsin was used because Pirie (1935) found that the lipoids in commercial trypsin, not the enzyme, inactivated vaccinia virus. The virus-trypsin mixture was then incubated at 37°C. in a water bath for from 30 to 50 minutes. The digested material was then centrifugalized in the angle centrifuge three additional times, resuspending in buffered water of pH 7.2 to 7.6. The final sediment was suspended in buffered saline, pH 7.4 to 7.6, and centrifuged 15 to 30 minutes at 3,000 R.P.M. in the ordinary horizontal centrifuge to throw down particles larger than the L.C.L. bodies. The opalescent supernatant fluid was then removed.

The use of trypsin for the digestion of tissue materials was first suggested by Nicolle and Adil Bey (1906), and was re-described by Smadel and Wall (1937). The method has been applied with considerable success by the latter workers to purify vaccinia suspensions. Other viruses which are relatively unaffected by the action of purified trypsin are Rous and Fujinami fowl tumors (Pirie (1935)) and swine influenza and equine encephalomyelitis (Merrill (1936a)), but pseudo-rabies virus is destroyed (Merrill (1936a)). Table 3 illustrates the failure of purified trypsin to inactivate psittacosis virus.

Tryptic digestion was an important procedure. Suspensions of virus after three washings still contained soluble tissue proteins, as indicated by a positive sulfosalicylic acid test, if trypsin was not employed. After two washings following tryptic digestion, the wash water was negative for soluble protein, by the same

test. Treatment with trypsin usually reduced the amount of the angle sediment to less than one-half its original volume. Smears of the angle sediment were much more free of amorphous material after digestion, when stained by Castaneda or the silver precipitation method of Morosow (1926).

Isotonic saline could not be employed as a washing fluid because the elementary body sediment underwent spontaneous agglutination in the presence of 0.85 per cent saline. Craigie and Wishart (1934b) have described the same phenomenon with vaccinia elementary bodies. The sediment from a saline suspension was granular and impossible to resuspend. The final suspension was made in buffered saline in order that the electrolyte concentration would be satisfactory for serological studies. These suspensions

TABLE 3

Illustrating lack of toxicity of trypsin

	294TH PASSAGE STRAIGHT	
Before trypsin	D2+	D4+
After trypsin	D2+	D3+
After washing	D4+	D4+

D = mouse dead, + = typical findings for psittacosis. Figures indicate days after intraperitoneal inoculation of 0.5 cc.

could be stored indefinitely in the ice chest with little sign of spontaneous agglutination.

Although tables 2 and 3 show the lack of toxicity of petroleum ether and purified trypsin in the procedures used, it was considered desirable to do a more complete titration which would give a comparison between the crude suspension and the same material following purification by the procedures described. Table 4 shows the result of such a titration. The material used was obtained from the 318th passage of egg membrane virus and was titrated by the usual method of intraperitoneal inoculation of 0.5 cc. into susceptible white mice.

Table 4 not only illustrates the close comparison between the infectiousness of the crude and purified suspensions, but also indicates the variation in individual susceptibility of mice receiv-

ing the same material. Also may be observed the tendency of mice receiving small amounts of virus to acquire a latent infection which may be proved only by autopsy. This latter property of the psittacosis virus has led to false interpretations of neutralization and immunization experiments in the past.

TABLE 4

Comparison of crude and purified suspensions

DILUTION	CRUDE		PURIFIED	
Straight	D2+	D2+	D3+	D3+
	D2+	D3+	D3+	D3+
10^{-2}	D4+	D4+	D4+	D7+
	D4+	D5+	D5+	D9+
10^{-4}	D7+	D9+	D7+	D9+
	D8+	D9+	D8+	S60+
10^{-5}	D7+	D15+	D7+	D9+
	D9+	S60+	D9+	S60+
10^{-6}	D10+	D22+	D11+	S60−
	D21+	D60−	D15+	S60−
10^{-7}	D2−	D19+	D10+	D2−
	D18+	S60+	D11+	D13+
10^{-8}	D9?	D12+	D11+	D19+
	D10+	S60+	D15+	S60+
10^{-9}	S60+	S60+	D10+	D23+
	S60+	S60+	D21+	S60−

D = mouse dead, S = mouse sacrificed, apparently healthy, + = typical findings for psittacosis, − = negative findings for psittacosis, ? = questionable findings anatomically. Figures indicate number of days after inoculation.

Standardization of purified suspensions

It was considered of value to attempt to standardize the L.C.L. body suspensions, since they were to be used as antigens and uniform dosage was desirable. Standardization by mouse titration was not possible, since the suspensions had poor keeping

qualities and the length of time needed to observe inoculated animals precluded the use of this method.

An attempt was made to compare suspensions of the type used with turbidity standards of the MacFarland type (Kolmer and Boerner (1938)). The no. 2 tube of this series, corresponding approximately to 6×10^8 bacteria per ml., was used throughout this study. Attempts to compare the L.C.L. suspensions directly to the nephelometer tube gave unsatisfactory results, due to the difference in optical properties. The virus suspensions were of an opalescent nature, as compared to the opaque barium sulphate standard. However, it was found that an infusorial earth suspension had similar optical properties to the L.C.L. suspensions, and a permanent standard was prepared from finely ground infusorial earth. This tube roughly resembled the MacFarland no. 2 nephelometer tube, and corresponded to between 2×10^9 and 4×10^9 L.C.L. bodies per ml., as will be shown in the direct counting method described below. This standard infusorial earth tube was used in the preparation of all L.C.L. bodies for antigenic purposes.

Since the turbidity standard gave only approximate results an attempt was made to determine the number of L.C.L. bodies more accurately. For this purpose the Petroff-Hausser counting chamber was considered most valuable, since it permitted the use of the darkfield together with the oil immersion objective. This chamber had a depth of 0.02 mm. and was equipped with an improved Neubauer ruling. The special reinforced coverglass permitted the use of the oil immersion objective and maintained the entire depth of the cell in sharp focus.

In this counting chamber, each square had an area of 2.5×10^{-3} mm. and a depth of 2×10^{-2} mm. This gave a total volume of 5×10^{-5} cu. mm. or 5×10^{-8} ml. per square. Each particle seen in one square therefore represented 2×10^7 particles in the suspension being used. This was corrected when diluted suspensions were being counted, so that the number of particles in the washed elementary body preparations was readily obtained.

Before making the count, the chamber was carefully cleansed, and all oil removed from the surfaces. All suspensions were

lightly centrifuged at 1,500 R.P.M. for 5 minutes before counting, in order that lint and debris would be eliminated. The suspensions counted were 1-in-10 dilutions of washed suspensions. All dilutions were made in double distilled water to which had been added 1 per cent formalin. These suspensions were incubated at 37°C. for 6 hours preceding the counts, in order that all the virus would be inactivated. As far as possible, all precautions were taken to prevent contamination of any part of the counting chamber other than that in actual contact with the killed virus suspension, and the entire chamber was placed in alcohol on completion of the count. Due to the presence of the formalin

TABLE 5

Distribution of particles in counting chamber

NUMBER OF PARTICLES PER SQUARE	SQUARES CONTAINING GIVEN NUMBER OF PARTICLES
12 or less	7
13	6
14	8
15	7
16	7
17	6
18	6
19 or more	3
Total	50
Mean	15.04

and the incubation period necessary to inactivate the elementary bodies, it was felt that the method probably was inaccurate, since some of the virus particles were undoubtedly lysed during the treatment. However, no other safe method presented itself and the counts were done only by the technique already described. The comparison between the number of virus particles and the infectiousness for white mice is discussed below.

The elementary bodies were readily identified in the counting chamber, using 10× oculars and a 1.9 mm. oil immersion objective. The counts were repeated several times. The results of one count which is considered typical of all are given in table 5.

This particular suspension therefore contained $15 \times 10 \times 2 \times 10^7$ or 3×10^9 particles per ml., since the counted material was diluted 1 in 10. This particular suspension also closely resembled the infusorial earth standard already described.

The Breed method as used for counting bacteria in milk was considered as a possible means for estimating the number of L.C.L. bodies in a washed virus suspension. A preparation was made by washing egg membrane virus the usual 3 hours in the angle centrifuge and placing a volume of 0.01 ml. of the resulting suspension in a measured area. The smear was dried and stained by the Giemsa method after fixation. The elementary bodies were counted and the total number estimated. Each field contained approximately 6,000 L.C.L. bodies, giving an estimated number of 3×10^8 particles per ml. Due to the obvious discrepancy with mouse titration and direct counts as already described, this method was discarded. It was impossible to secure a uniform distribution of the particles in the measured area, the bodies tending to be far more concentrated at the edges of the smear. In addition, there was a known loss of virus during the staining process and the Breed count method was considered to be unsatisfactory for standardization purposes.

It was observed that the number of elementary bodies in a freshly prepared suspension gave a good indication of the infective titer of that suspension. That is, a suspension containing 3×10^9 elementary bodies was usually infectious in a dilution of 10^{-9}. This fact immediately suggested that the number of elementary bodies required to infect a susceptible white mouse was exceedingly small. Although the available evidence pointed toward an infection with one elementary body, no statistical methods could be successfully applied to determine the exact number of L.C.L. bodies required. Preliminary titration was impossible, due to the time required to infect mice if high dilutions were used and also because of the poor keeping qualities of the washed virus suspensions, as discussed below. An accurate statistical analysis could only be made by using several hundred mice and it was felt that the results did not warrant such an expensive procedure. The small number of elementary bodies needed to infect is of

interest in view of the recent work showing that only one Paschen granule is needed to infect the skin of a rabbit (Parker and Rivers (1936), Parker (1938)). Merrill (1936b) has also shown that the minimal infective dose of equine encephalomyelitis virus for white mice is less than 100 particles and probably less than 10 particles.

Since it was decided to use the purified L.C.L. body suspensions as antigenic material, it was necessary to investigate the keeping properties of the washed virus when stored in the ice chest at $+4°$. It was soon observed that this material rapidly lost its infectiousness, and a washed and purified suspension was shown to be non-infectious for white mice 29 days after preparation and storage. This material had originally been infectious in a dilution of 1×10^{-8}. There was little observable difference in the keeping qualities of the washed virus whether it was suspended in buffered water or in buffered saline. The non-infectious suspensions were perfectly satisfactory for agglutination tests, as will be shown in a later section.

SUMMARY

1. The chorio-allantoic membrane of the developing egg has been used as a source for large amounts of psittacosis virus.

2. The elementary bodies of psittacosis were freed of soluble protein and concentrated by means of washing, tryptic digestion and differential centrifugalization. These procedures did not result in an appreciable inactivation of the virus.

3. The L.C.L. body suspensions were standardized by direct counting of inactivated suspensions in a darkfield counting chamber and by comparison with an infusorial earth standard.

4. Both the crude and washed suspensions were found to lose their infectiousness for white mice after short periods of storage in the ice chest.

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THE VIRUS OF PSITTACOSIS

II. Centrifugation, Filtration and Measurement of Particle Size[1]

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In a previous communication (Lazarus and Meyer (1939)), the propagation of the psittacosis virus and the preparation and standardization of washed suspensions have been discussed. In this paper, some of the uses to which these materials have been put will be outlined.

CENTRIFUGATION

Review

Bedson and Western (1930) centrifuged filtrates of Chamberland L1 bis candles for 2 hours at 5,000 R.P.M. The original material was mouse spleen ground in buffered water. Titration of the uncentrifuged material, the supernatant and the sediment in guinea pig skins showed a definite concentration of the virus in the sediment. No data are supplied regarding the temperatures reached during the centrifugation. Further experiments by Bedson (1932) showed that mouse spleen suspensions, after a preliminary centrifugation to remove gross particles, yielded a concentrated elementary body deposit after 2 hours at 5,000 R.P.M. In addition, by washing and fractional centrifugation the virus was freed from extraneous protein. Stained smears of the final deposits showed that the only significant particulate material present was the elementary bodies.

[1] Portion of a thesis submitted by the senior author in partial satisfaction of the requirements for the degree of Doctor of Philosophy, University of California.

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Numerous workers have been successful in concentrating the elementary bodies of vaccinia by the use of the angle centrifuge. These elementary bodies are probably slightly smaller than those of psittacosis. Craigie and Wishart (1934) describe the technique of centrifugation, and the results reported in the literature leave no doubt that a concentration and sedimentation of the vaccinia bodies has been accomplished. These findings have also been applied to the concentration of psittacosis virus, as already reported in previous sections of this paper.

In view of the results reported above, it was considered of interest to determine whether the psittacosis virus could be completely sedimented and washed, and whether the supernatant fluid could be freed of virus. No previous work of this nature is available as far as egg membrane virus is concerned.

Experimental technique

The ordinary type of laboratory centrifuge attaining a speed of 3,000 revolutions per minute was used for removal of gross tissue debris in the preliminary preparation of the virus for the following studies. The angle centrifuge used in these experiments had a maximum speed of 3,000 R.P.M. It held 12 tubes at an angle of 30° from the vertical. The flat tubes had a capacity of 4 ml. each.

The high speed apparatus was an International Size 1, Type SB centrifuge equipped with a multispeed attachment and a number 295 conical head. The head had a capacity of 6 tubes held at an angle of 45° and containing approximately 4 ml. each. The machine was capable of attaining a speed of 20,000 R.P.M., but for this study the speed was kept between 12,000 and 15,000 R.P.M. so that results comparable to those already reported with vaccinia could be obtained. Ordinary glass tubes were frequently pulverized during the high speeds attained by this machine, and considerable difficulty was experienced until a supply of heavy-walled Pyrex centrifuge tubes were specially made by the Corning Glass Works. These tubes were 60 mm. long, having an inside diameter of 11 mm. and an outside diameter of 15 mm. They had a round bottom and a capacity of slightly less than 4 ml.

The high speed centrifuge attained a temperature of 40°C. after a run of 30 minutes or longer and since the virus of psittacosis is inactivated rather easily at high temperatures, some procedure was needed to control this factor. Since the conical head of the high speed centrifuge did not rotate in the middle of the protective case, but close to one side, it was possible to add approximately 15 pounds of dry CO_2 ice without affecting operation of the machine. By using about 10 pounds of dry ice per hour, the temperature of the interior of the centrifuge was kept well below 16°C. and no trouble was experienced with virus inactivation.

The use of gelatin or agar gels for the purpose of preventing redispersion of sedimented particles has been approved by workers in the virus field. A disc of thick filter paper has likewise been successfully used for this purpose by Schlesinger (1932). For purposes of this study, a solution of 20 per cent gelatin was rapidly sterilized in the centrifuge tubes at 20 pounds pressure in the autoclave and the tubes were rotated as they cooled, giving a uniform layer of gelatin around the lower half of the tube.

Results

Space prohibits publication of the complete protocols for this series of experiments. Fifteen separate experiments were planned and carried out. Crude suspensions, prepared as already described (Lazarus and Meyer (1939)), were subjected to centrifugation from 1 to 8 hours, at speeds ranging from 3,000 to 14,000 R.P.M. Suspensions of the virus were tested before and after centrifugation, and both supernatant fluid and sediment were tested when practicable. Titrations of the material obtained were made in the usual manner by intraperitoneal inoculation of 0.5 ml. into susceptible white mice. All material was inoculated within 3 hours of the end of the experiments and the suspensions to be tested were held in the ice chest until animal injection was done. All sediments were resuspended in the original volume of fluid so that titrations were comparable. Sampling was done as carefully as possible with a capillary pipette controlled by a rubber teat. Care was taken not to touch the sides of the tube or to disturb the sediment in any way.

Table 1 gives in a condensed form the results of representative experiments. The titer given shows the highest significant positive result obtained, although the end-point was not reached in all cases. All dilutions were made in infusion broth at pH 7.2 to 7.6.

Results similar to those reported in table 1 were obtained when the angle centrifuge was used at a speed of from 3,000 to 4,500 R.P.M. While the sediment contained large numbers of L.C.L. bodies, the supernatant was also highly infectious when carefully removed and tested by mouse inoculation. There was no doubt that much virus was discarded in the washing processes described in the purification experiments.

TABLE 1

Results of centrifugation experiments

STRAIN	PASSAGE NUMBER	MENSTRUUM	R.P.M.	TIME	SEDIMENT	SUPERNATANT	CONTROL
				hours			
Maine	14	Buffered H_2O pH 7.0	12-14,000	1	10^{-6} K12+	10^{-3} D3+	10^{-4} K6+
Maine	17	Buffered H_2O pH 7.0	13-15,000	1	10^{-3} K6+	10^{-3} D8+	10^{-2} D6+
Maine	35	Buffered saline pH 6.9	12,000	3	10^{-2} D7+	10^{-3} D9+	10^{-2} D9+
Maine	52	Buffered H_2O pH 7.7	12,000	3	10^{-3} D5+	10^{-3} D9+	10^{-6} D9+
Maine	57	Buffered H_2O pH 7.3	12,000	6	10^{-2} D6+	10^{-2} D10+	10^{-8} D12+
Maine	70	Buffered saline pH 7.4	13,000	8	0	10^{-4} D10+	10^{-4} D10+

K = mouse killed in *extremis*, D = mouse dead, + = positive findings for psittacosis, 0 = not done. Figures indicate number of days after inoculation.

Discussion

From the preceding data, it appears obvious that the virus of psittacosis could not be entirely removed from a fluid suspension under the conditions of these experiments. The use of fuller's earth as an adsorbing agent gave no indication of more satisfactory sedimentation. The use of gelatin-coated tubes gave slightly better results, but in no case was there any indication that a suspension could be entirely freed from infective particles.

Prolonged centrifugation of 6 to 8 hours at high speeds showed some indication of more complete sedimentation. It was possible that a much longer period would gradually deplete the supernatant fluid of virus, but obviously such methods were impracticable.

In view of the previously reported successes with psittacosis and vaccinia virus concentration using animal material, it is possible that the chick membrane virus was physically different from material from other sources.

Physical factors may have been involved to a great extent in the centrifugation methods herein reported. As pointed out by Elford (1936), variations in temperature between different parts of the liquid will give rise to convection currents and so prevent uniform motion of the sedimenting particles. Since the tubes of the high speed centrifuge were completely enclosed in a head which spun in a cooled atmosphere, the conducted heat from the motor and shaft may have set up a temperature gradient in the liquid. However, the shaft was likewise exposed to the chilled air, and this factor probably played a minor part in these experiments.

Elford (1936) has likewise pointed out that mechanical vibration may tend directly to stir up a sediment if it is 'not tightly packed. In addition, indirect vibration transmitted to the liquid air interface may be carried into the liquid and, if not damped out before reaching the bottom of the tube, will tend to be reflected and disperse any sedimented particles. That this factor played an unimportant part in the preceding studies is shown by the fact that the gelatin-coated tubes gave no better results than the plain tubes. In addition, the centrifuge itself was quite steady while the speed was constant and gave only a minimum of vibration while starting and stopping.

The process of sampling probably contributed greatly to the failure to sediment the virus particles completely. In the preceding series of experiments, sampling was done by hand, using capillary pipettes controlled by a rubber teat. Elford (1936) states flatly that "satisfactory sampling by hand is quite impcssible." He recommends the use of a capillary pipette raised and lowered by a rack-and-pinion movement, with the sample taken by the use of a rubber teat controlled by a screw clamp. It is probable that such a process would have given more favorable results in the previous studies, but it appears that this factor alone could not have been responsible for the lack of complete sedimentation.

SUMMARY

1. Centrifugation of psittacosis chick membrane virus suspensions for prolonged periods at high speeds has failed to yield complete sedimentation of the infective agent.

2. Adsorbing agents and gelatin-coated tubes have not aided in complete sedimentation of the virus particle.

3. The virus of psittacosis could be concentrated and washed by centrifugation methods, but large amounts of virus were lost by the technique used.

FILTRATION

Berkefeld, Chamberland and Seitz EK—review

The literature regarding the filtration of material containing psittacosis virus is still more or less confused and variable results have been reported. The consensus of opinion is that the virus can pass through the more porous candles, such as the Chamberland L1 bis, L2 and the Berkefeld V, while filters with smaller pores hold back the virus in much higher degree. As pointed out by Elford and Andrewes (1932), the findings in vaccinia filtration experiments are likewise irregular, while Blaxall (1930) has emphasized the confusing evidence in filter-candle work with vaccinia material. Gordon (1925) mentions the variability of filtration of vaccinia and variola material through Berkefeld candles, and recommends the storage of virus-containing material in the ice box for several weeks. The resulting cell autolysis aids in filtration, since the virus is freed from the cells. These data all point to the close resemblance in size of the viruses of psittacosis and vaccinia, a theory amply supported by the microscopic measurements of elementary bodies from the two infections.

Coles (1930) reported finding coccoid bodies in the psittacosis filtrates of Seitz pads, while Gordon (1930) passed infectious material from two birds and two human cases through Seitz filters and obtained positive results, with, however, some loss of the virus. Krumwiede (1930) reported positive results with Berkefeld V candles, and Bedson, Western and Simpson (1930a, 1930b) likewise obtained infectious filtrates through Chamberland

L1 bis and Seitz EK filters, using budgerigars as test animals. Bedson and Western (1930) used Chamberland L1 bis and L2 candles and Seitz EK pads and reported some loss of virus when the filtrates were titrated in the guinea pig skin. Sacquépée and Jame (1930) reported some loss of the virus through Chamberland L3 candles, while Levinthal (1930a) obtained some positive and some negative results using Berkefeld V candles. Elkeles and Schneider (1930) obtained positive filtration using Berkefeld N and V candles, but reported some loss of virus through Reichel D filters. Levinthal (1930b) obtained infectious filtrates from Berkefeld V candles with an estimated pore size of 3.8 μ, while the same worker obtained a non-infectious filtrate through a Berkefeld W filter with an estimated pore size of 1.9 μ. Pesch (1930) reported no noticeable difference between filtered and unfiltered material using Seitz EK, Chamberland L2 and L3 and Berkefeld V candles. Armstrong, McCoy and Branham (1930) likewise found no material difference between filtered and unfiltered material using Berkefeld N candles.

The data supplied with all these filtration experiments are uniformly sketchy. No information is available as to the pressures used, the suspending medium, the hydrogen ion concentration or the time and temperature of filtration. Whether or not the candles were new has considerable bearing on the results, as will be shown in the following data. Without further information, an accurate analysis of the data reviewed above was impossible and it was decided to attempt various filtration experiments under carefully controlled conditions.

Experimental technique

The general procedure for the preparation of psittacosis suspensions has already been outlined (Lazarus and Meyer (1939)). The suspensions used in the filtration experiments were of the crude type and the filter candles were standard. Suction for these experiments was supplied by an ordinary water pump, except in the mentioned cases where a higher vacuum was obtained from an electric pump. All filtrations were carried out at room temperature (19 to 23°C.). Egg membrane virus was used

exclusively for these filtration experiments and controls were consistently used, in spite of the unaltered virulence of the membrane virus after numerous passages. All suspensions were prepared from 72-hour membranes, and routine Castaneda smears were made both on the original material and on the mice as they succumbed to the infection.

All material tested was injected intraperitoneally into white mice of a common stock with a uniform susceptibility proved by previous experiments. Filtrates were held in the ice box for a period not exceeding 4 hours before inoculation.

The complete protocols are too lengthy for publication. Table 2 summarizes the results in composite form.

TABLE 2

Composite summary of filtration through candles and Seitz pads

EGG VIRUS MENSTRUUM	NEW BERKEFELD V	USED BERKEFELD V	BERKEFELD N	BERKEFELD W	NEW CHAMBERLAND L3	SEITZ EK
Buffered H_2O pH 7.0......	∓ (2) − (2)	− (1) ∓ (2)	− (1)	− (1)	∓ (2) − (1)	∓ (1) − (1)
Buffered H_2O pH 8.0.......	0	− (1)	0	0	− (1)	− (1)
Buffered saline pH 7.4.....	0	− (1) ∓ (1)	0	0	0	− (1)
Hormone broth pH 7.8.....	0	∓ (1)	0	0	0	0

∓ = small amount of virus in filtrate, − = virus absent from filtrate, 0 = not done. Numbers in parenthesis represent times done.

Discussion of results

The most striking observation which can be made concerning the preceding experiments is the great loss of virus which accompanied filtration through candles and Seitz pads. Control animals succumbed up to a dilution of 10^{-6} or higher with regularity, but even in those filtrations which showed some positive results, a great diminution of the amount of virus was obvious, the titer rarely exceeding 10^{-3}. It appears clear that on a quantitative basis, successful filtration through filter candles and Seitz pads did not occur under the conditions of these experiments.

The results, using the same materials and the same types of candles, could not be repeated under the same approximate condi-

tions, showing the extreme variation found when working with this form of filter. Seitz pads gave consistently negative results, with one inconclusive exception. Chamberland L3 candles gave negative results in 2 out of 4 attempts, but one of the positive findings was obtained only after the use of a high vacuum over a long period of time. Berkefeld N and W candles gave no positive results. One Berkefeld V candle showed 3 positive filtrations out of 4 attempts with, however, a great loss in the virulence of the filtrate as compared with the original unfiltered material. Two other Berkefeld V candles showed 1 positive filtration and 1 completely negative, while the fourth and fifth candles of this type gave no suggestion of virus in the filtrate.

Through the kindness of Dr. A. P. Krueger, the electrical charge was measured on 5 of the Berkefeld candles used. Dyes with colored cations were passed through the candles and the point at which the negative charge of the candles was satisfied was determined by the presence of color in the filtrate. The candles removed a large amount of dye from solution, which accounted for the retention of virus by this type of filter. Saturation of the candle surface by acidulated egg albumen satisfied the charge of the candle and improved the filtration.

Lacking details of the experiments reported in the literature, no comparison could be made with the work already reviewed. It is conceivable that the virus could pass through a used filter candle under extreme conditions of vacuum and time, but in view of the results herein reported, the virus of psittacosis experienced great difficulty in passing through ordinary filters under the conditions of the experiments. What virus was found in the filtrate was greatly reduced, in proportion to the original infectiousness of the material. Since early reports dealt only with the qualitative presence of virus in the filtrate, without any attempt to titrate the amount present, it appears probable that small amounts were forced through the filters used and, due to the extremely high infectiousness of this virus, the results of filtrate inoculation suggested satisfactory filtration.

The literature contains no reference to filtration with egg membrane virus. In view of the larger size of the elementary

body in this medium, as previously described, and the difficulty experienced in filtration work, it might be postulated that the infective particle had actually increased in size. This viewpoint was not supported by the centrifugation and ultrafiltration findings. It is reasonable to assume that the cultivation of this virus in the developing egg had resulted in the deposition of a coating around the infective particle, which may have accounted for the inability to pass through filters, as well as the increased particle size by the Castaneda stain.

Ultrafiltration—review

The only mention in the literature regarding the ultrafiltration of psittacosis virus is in the paper of Levinthal (1935). No details regarding the conditions of filtration are available. Sir Henry Dale (1935) also refers briefly to unpublished experiments of Elford.

Experimental technique

The ultrafilters used were of the Elford "gradocol" type. Complete details of the original method of Elford have been published (Elford (1931)) and a simplification of the method has been proposed by two American workers (Bauer and Hughes (1934)).

In practice, the Bauer and Hughes modification of the Elford technique adds acetone and amyl alcohol to the ether and ethyl alcohol used for dissolving the nitrocellulose. Either acetone or amyl alcohol in conjunction with ether and ethyl alcohol constitutes an excellent solvent for nitrocellulose, but both together, in certain concentration, act antagonistically toward each other and initiate a coagulation and precipitation of the nitrocellulose in solution. When the collodion mixture is poured out and the solvents allowed to evaporate, the concentration of the least volatile ingredients becomes progressively increased. Thus amyl alcohol, being the least volatile of the components, becomes relatively more concentrated during the evaporation process, and in the presence of acetone initiates an aggregation of the nitrocellulose while the residual ether and alcohol maintain a sponta-

neous gelation process. Hence both coagulation and gelation processes take place during the evaporation until the entire membrane is immersed in water, which serves to fix the potentially existing structure and to replace the remaining solvents. The resulting membranes are highly permeable and have considerable tensile strength. Elford also found that the addition of small amounts of glacial acetic acid reduces the extent of coagulation taking place during the period of evaporation and results in less porous membranes, while the addition of water hastens the coagulation and results in membranes of greater

TABLE 3

Composite table of ultrafiltration results

VIRUS SOURCE AND MENSTRUUM	AVERAGE PORE SIZE OF MEMBRANES USED					
	.645μ	.454μ	.436μ	.410μ	.383μ	.288μ
Mouse organs hormone broth	+ (1)	+ (1)	+ (1)	+ (1)	− (1)	0
Egg membrane virus hormone broth	+ (2)	+ (1)	+ (1)	+ (2)	− (1) ∓ (1)	− (1)
Egg membrane virus buffered water	0	0	0	+ (1)	− (1)	0
Egg membrane virus buffered saline	+ (2)	0	0	0	0	0

+ = virus in filtrate same virulence for susceptible white mice as unfiltered controls, − = virus absent from filtrate, ∓ = diminished amount of virus in filtrate, 0 = not done. Numbers in parenthesis = number of times done.

porosity. The final products, after prolonged washing to remove the relatively insoluble amyl alcohol residue, have been termed "gradocol" membranes by Elford, since they are products of a graded coagulation of collodion.

The technique of Bauer and Hughes was used in preparing the membranes for the ultrafiltration studies, and yielded highly satisfactory results. All ultrafiltration was carried out at room temperature, using a positive pressure of high purity nitrogen ranging from 18 to 30 cm. mercury. The volume of filtrate ranged from 5 to 12 ml. and all filtrations were complete in 4 minutes or less. The membrane thicknesses ranged from 0.136 to 0.152 mm. The use of a 1 in 100 dilution of the original crude

suspension obviated the necessity of a preliminary filtration to remove gross tissue particles. *Serratia marcescens* was added routinely before filtration and could not be demonstrated in any filtrates. All membranes were satisfied by the preliminary passage of 5 ml. of the diluting fluid.

Table 3 summarizes the results of ultrafiltration experiments in a composite form.

Discussion of ultrafiltration studies

The most noteworthy observation to be made as a result of membrane filtration was the highly satisfactory recovery of virus in ultrafiltrates, as compared to the almost complete retention by filter candles and Seitz pads. Using membranes of the Elford type, the virus of psittacosis consistently passed through with unaltered infectiousness for mice, even in membranes whose porosity closely approached the end-point of the virus particle. These results demonstrated most conclusively the vast superiority of the graded collodion membrane over any other type of filtration used in this study. Bacteria could be removed from contaminated virus suspensions and a filtrate containing all the original virus could be obtained consistently. Low pressures were used and the ease and speed of this method of filtration cannot be emphasized too strongly. No bothersome cleaning process was necessary, since the collodion filters were used once and discarded. Results were completely reproducible. Both buffered saline and broth suspensions yielded satisfactory ultrafiltration results, with the broth apparently giving slightly superior results. This is in accord with the findings of other workers (Ward and Tang (1929), Elford (1933)).

It was obvious that pore diameter alone did not determine the degree of virus recovery from the filtrates. Berkefeld candles with a pore size of from 5 to 7 μ gave unsatisfactory filtration of psittacosis virus, with complete or almost complete retention of the virus. On the other hand, using the same type of suspension and the same method of testing the filtrates, collodion membranes with an average pore size of 0.6 μ or even less, gave filtrates which contained as much virus as the original material, with the added

advantage that all bacteria, tissue debris and detritus had been removed. The ease of filtration and the complete reproducibility of results make it obvious that the collodion type of membrane filter is the most satisfactory method available for this type of work.

SUMMARY

1. The infective particle of psittacosis was completely, or almost completely retained by Berkefeld V, W and N and Chamberland L3 filter candles and Seitz EK filter pads, using chick membrane virus.

2. The failure to confirm the filtration results of other workers was investigated and explained on the basis of a lack of quantitative titration of the virus by previous investigators.

3. The satisfactory results of ultrafiltration studies with psittacosis virus have been demonstrated and the superiority of this type of filtration has been emphasized.

MEASUREMENT OF VIRUS PARTICLES

Review

Lillie (1930) estimated the elementary body of psittacosis to be from 0.2 to 0.3 μ in diameter, using direct microscopic measurements of stained preparations. Coles (1930) stated that the smallest elementary bodies measured from 0.24 to 0.3 μ, using photomicrographic measurements. Levinthal (1935) using the "gradocol" membranes of Elford, stated that the minimum particle size was from 0.22 to 0.33 μ, but gave no data on the methods of filtration or the type of material used. Sir Henry Dale (1935) stated that Elford has found the virus particle size to be 275 mμ, but this material has not yet been published by Elford and no data are available. In view of the incomplete material in the literature, it was considered of value to attempt a measurement of the virus particle under controlled conditions.

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Various authors have outlined tables of correction factors to be applied to ultrafilter pore sizes to obtain a range of true particle size. This aspect has been discussed notably by Krueger and Ritter (1930), Bechhold (1931) and Elford (1933). These workers have taken into consideration the various factors which tend to influence the filtration of a small particle and have made a mathematical attempt to supply empirical corrections to apply to the filtration end-point to secure true particle size. The need for this correction can readily be seen when the physical factors entering into the passage of a small particle through a pore are considered. When it is realized that the pore length to pore width ratio cannot fall below 1 to 3,000, the necessity for correction will be apparent, and in calculating the size of the particle from the

TABLE 4

Relation of size of retained particle to average pore diameter of gradocol membranes (Elford 1933)

MEMBRANE AVERAGE PORE, DIAMETER	SIZE OF RETAINED PARTICLE
$m\mu$	
10–100	(0.33–0.5) d
100–500	(0.5 –0.75) d
500–1,000	(0.75–1.0) d

d = average pore diameter of limiting membrane for optimum filtration conditions.

average pore diameter through which it just fails to pass, the effect of adsorption has to be considered. This effect is most influential in membranes with very small pores, as is shown in table 4.

Table 4 has been worked out by Elford as especially applying to the gradocol membranes he has described. Since the ultrafiltration results of this study were obtained with membranes of the Elford type, the correction factor as outlined above will be applied.

The estimated end-point of the psittacosis ultrafiltrations as summarized in table 3 is in the vicinity of 0.400 μ. Using this figure and applying the correction factor of 0.5 to 0.75 suggested by Elford, the figure arrived at gives the psittacosis particle a

size of 0.200 to 0.300 μ. This figure corresponds closely to the ultrafiltration results already reviewed. A brief summary of this work has recently been published (Lazarus, Eddie and Meyer (1937)).

Photomicrographic measurements

With the intention of verifying the ultrafiltration results as well as the published reports of other workers, an attempt was made to measure the diameter of the L.C.L. body by means of enlarged photomicrographs.

Smears of infected material from various hosts were prepared and stained by the various methods already described. Under optimum conditions of microscopic examination, a magnification of 900 times was used. Photomicrographs were prepared using a miniature Contax Phoku camera and panatomic film. After developing and examining the negatives, the most satisfactory were carefully enlarged. Measurement was accomplished both by means of knowledge of the amount of enlargement and by comparison with a 10 micra scale enlarged in the same proportion as the virus preparations.

The largest elementary bodies by this method measured 380 mμ, the smallest 280 mμ. The slightly larger bodies by photomicrographic methods as compared with ultrafiltration methods may be accounted for by a deposition of stain on the particle, by the multiplication of errors resulting from such high magnifications, or by a combination of both these factors. Due to the highly satisfactory results with ultrafilters of the Elford type, as confirmed by numerous workers, the ultrafiltration measurements were considered more reliable than the relatively crude photomicrographic measurements.

Discussion

The ultrafiltration and photomicrographic measurements correspond within the limits of error inherent in such methods. These figures, strengthened by the results of candle filtration and centrifugation experiments, lead to the logical assumption that the infective particle in psittacosis is either the elementary body,

a submicroscopic particle not separable from the elementary body, or an invisible particle of the same order of size as the elementary body. The first statement appears to be the most logical and this interpretation is well supported by the work showing the Paschen bodies of vaccinia to be the actual infective agent of that disease (Parker and Rivers (1936), Parker (1938)).

SUMMARY

1. On the basis of ultrafiltration measurements, as verified by photomicrographic studies, the infective particle of psittacosis was found to have a minimum size of 0.200 to 0.300 μ, a figure in close agreement with that obtained by other workers.

2. The infective agent of psittacosis was shown to be the elementary body itself, a substance not separable from the elementary body, or an invisible particle the same size as the elementary body. The first theory is shown to be the most probable, based on results of centrifugation, pad and candle filtration, and ultrafiltration.

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THE VIRUS OF PSITTACOSIS

III. Serological Investigations[1]

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In previous communications (Lazarus and Meyer (1939a, 1939b)) the virus of psittacosis has been investigated with respect to its filterability and general properties. The present report deals with the serological reactions which could be elicited with the washed and concentrated suspensions of elementary bodies.

AGGLUTINATION

Review

The literature contains little reference to serological studies on the psittacosis virus, with the exception of work concerning the complement fixation reaction, which has been extensively investigated by Bedson (1933). Since the complement fixing antibodies were not considered in this study, no further reference need be made to this phase of the subject.

Bedson (1932) attempted to produce antipsittacosis sera in guinea pigs and mice, with some success. Agglutination tests performed with twice-washed mouse elementary bodies and antiserum from guinea pigs gave positive agglutinations in dilutions of 1 in 8. The reaction was shown to have been specific, but such low titers are rather unsatisfactory. In the same study, neutralizing antibodies were demonstrated in an equally low dilution. No reference was made to precipitin tests, and no

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attempts have been described employing neutralization of the virus on the chorio-allantoic membrane of the chick.

The particular problems involved in the virus-antibody reaction must be only special examples of principles applicable to all antigen-antibody reactions. The modern trends of thought concerning these reactions are well expressed by Marrack in "The Chemistry of Antigen-Antibody Reactions" (1938). The problem of applying these general concepts to viruses has been extensively discussed by Burnet, Keogh and Lush (1937) in their comprehensive monograph.

In view of the demonstration of two antigenic groups in the vaccinia elementary body (Craigie and Wishart (1934)) and the presence of at least 3 different types of foot-and-mouth virus (Topley and Wilson (1936)), there is reason to believe that some virus particles, at least, may closely resemble the bacteria in respect to antigenic properties in general. The demonstration of a polysaccharide component in vaccinia elementary bodies has further verified these views (Craigie (1932, 1935)).

For technical reasons the study of the *in vitro* aggregation reactions of vaccinia elementary bodies has advanced much further than that of any other animal-pathogenic virus. The important preliminary work was accomplished by Gordon (1925), Burgess, Craigie and Tulloch (1929) and Craigie and Tulloch (1931), although Paschen (1913) had observed the elementary bodies many years previously. The method of Craigie (1932) rendered it practicable to obtain large amounts of crude material very rich in the elementary particles of the virus. In general, the principles outlined in the above studies have been followed in the investigations of psittacosis virus.

Schultz, Bullock and Lawrence (1928) claimed that the agglutinations and precipitations observed in vaccinia studies were the result of the presence of bacteria, both in the antigenic material and in the reagents used in the actual tests. This theory has been disproved by the careful investigations of Craigie and Tulloch (1931) and has been completely discredited by the use of bacteria-free elementary body suspensions by Smadel and Wall (1937). Sterility tests with psittacosis ele-

mentary body suspensions were completely negative and no account need be taken of the possible influence of bacteria.

In addition to the studies of vaccinia agglutinations, work along these lines has also been reported for varicella and zoster (Amies (1934)), for Rous tumor virus (Ledingham and Gye (1935)) and myxoma (Rivers and Ward (1937)).

Experimental technic

The purified suspensions of L.C.L. bodies previously described (Lazarus and Meyer (1939a)) were used in the principal investigations of agglutination reactions. The determination of agglutination titers of animals receiving other types of psittacosis suspensions as antigenic material will be referred to in a later section.

The agglutination reaction in guinea pig sera was first investigated. Normal guinea pigs of approximately 300 grams weight were given 0.5 ml. of washed elementary bodies by means of intraperitoneal injections. This dose was approximately 1.5×10^9 active elementary bodies. The first inoculation was accompanied by a slight elevation of temperature (to about 104°F.) and a loss of appetite for about 48 hours, but this reaction was not observed after subsequent injections. The same apparent malaise has been observed by Bedson (1933). One week after the first inoculation, the animals were given 1 ml. of a fresh suspension of washed elementary bodies (approximately 3×10^9) by the same intraperitoneal route, and subsequent inoculations were made at intervals of from 5 to 8 days.

The guinea pigs were bled by cardiac puncture, the sera allowed to separate overnight in the ice chest and removed, centrifugalized and stored without preservative. Normal serum was collected before the first inoculation.

The technic of the agglutination test was similar to that described by Craigie (1932). Pyrex test tubes with outside dimensions of 10×75 mm. were carefully washed and then sterilized by dry heat. As a diluent in the tests, buffered saline, prepared as already described (Lazarus and Meyer (1939a)), was used.

This saline was made with freshly prepared double distilled water, and was autoclaved just before use. For agglutination, a relatively light suspension of elementary bodies yielded the most satisfactory results, and the stock suspension diluted to contain approximately 1×10^9 elementary bodies per ml. was found to be most satisfactory. 0.25 ml. of the different dilutions of immune sera were placed in the tubes, using sterile 1 ml. pipettes and aseptic technic. Saline and normal serum controls were used throughout. To the tubes was then added 0.25 ml. of the elementary body suspension. The same results were obtained whether the elementary bodies were freshly prepared, or were held in the ice chest long enough to cause them to lose their infectiousness for mice, as has already been discussed. All glassware used in the tests was subjected to careful washing and neutralization.

The tubes with their contents were placed in an ordinary serological rack and the mouths of the tubes were protected by a piece of sponge rubber sterilized by autoclaving. This in turn was covered with a block of wood secured in place by large rubber bands. This procedure prevented evaporation during the prolonged incubation period and also prevented the possibility of the virus particles being allowed to dry and be carried into the atmosphere by air currents. Cotton stoppers did not prevent evaporation and resulted in lint in the tubes, while small individual rubber stoppers were too awkward and difficult to manipulate. The covered racks were then incubated at a temperature of 46 to 48°C. for 20 to 36 hours.

Results

When agglutination reactions were carefully conducted in the manner described, convincing and consistent results were obtained. The results were easily read with the naked eye, although a magnifying lens was often of service in determining the exact end-point of the titration. Care had to be taken not to shake the tubes too violently, because agglutinated elementary bodies were almost completely dispersed by excessive agitation, and further sedimentation required long periods of time. The

same phenomenon has been observed with vaccinia elementary bodies (Parker and Rivers (1935)). The chick membrane elementary bodies when agglutinated by guinea pig sera clumped in a granular form, and complete sedimentation was observed

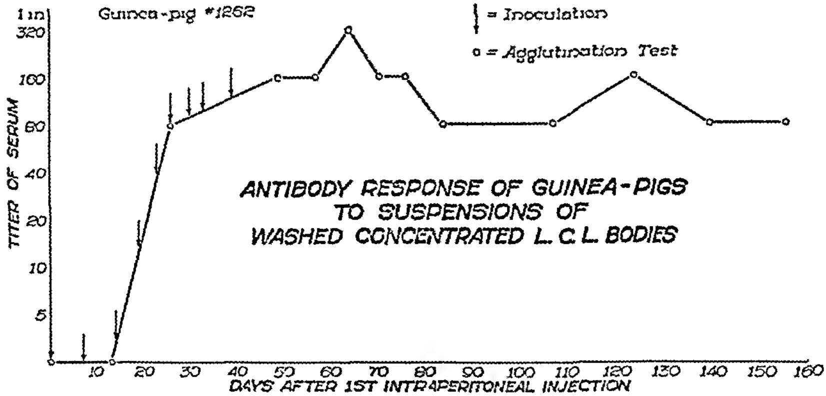

Fig. 1. Result of 9 Intraperitoneal Injections

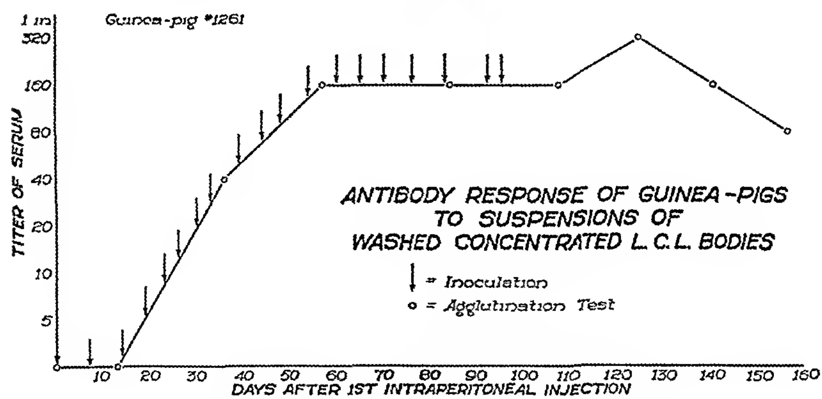

Fig. 2. Result of 19 Intraperitoneal Injections

only in the lowest dilutions. When sera from rabbits was used, large loose flakes were formed and the supernatant fluid was often completely clear. This point will be discussed in later sections.

The progress of agglutinin production being of considerable interest, an attempt was made to follow the rise and fall of agglu-

tinins in immunized guinea pigs. Figures 1 and 2 represent two typical curves produced by long continued injections of washed elementary bodies. All doses, represented by arrows, consisted of approximately 3×10^9 freshly isolated and washed elementary bodies, except the initial inoculation, which contained only half as many L.C.L. bodies. There were no symptoms resulting from these massive doses of highly infectious material, except for the slight rise in temperature previously mentioned as occurring after the first inoculation.

It will be immediately observed that although these sera were far superior to those obtained by Bedson (1933), no rise in titer over 1 in 320 could be induced, in spite of long continued inoculation with active material (fig. 2). In fact, other animals showed the same production of agglutinins following only 2 or 3 injections. The maintenance of a titer of 1 in 80 after discontinuance of injections is also noteworthy.

The failure to raise the titer above 1 in 320 is closely analogous to the results obtained using bacteria as antigens. It is well known that when the titer of any particular bacterial antibody has been forced up to a certain level, which of course varies widely with the nature of the antigen and the responsiveness of the animal injected, it becomes impossible to induce any further rise in the concentration of antibody in the circulating blood. This fact was pointed out as early as 1901 (Goldberg (1901)), and its application to the antigenicity of L.C.L. bodies lends further credence to the rapidly growing conviction that the elementary bodies are no more than highly parasitic organisms requiring an intracellular habitat for survival and reproduction, and lacking the necessary enzyme systems for independent existence.

Effect of incubation time and temperature

In the thorough investigations of Craigie (Craigie and Tulloch (1931) Craigie (1932)), the necessity for relatively long periods of incubation to elicit maximum agglutination of vaccinia elementary bodies was discussed. Similar results have been observed in studies of psittacosis material. It appears logical that

the incubation period must be longer than that required for bacterial agglutination, not because of any peculiarity inherent in the elementary bodies themselves, but because the particles and the clumps they form are much smaller than those resulting from bacterial agglutinations, and the time required to produce a visible reaction is therefore longer. This view is strengthened by the observation that psittacosis agglutination tests gave the same results after 60 hours whether the 48° incubation period lasted through the first 36 hours or only through the first 12 hours. Obviously, the higher temperature gave the usual result in the first few hours, and the visible reaction came about at any

TABLE 1

Effect of time and temperature on agglutination

DILUTION OF ANTISERUM	TIME OF INCUBATION IN HOURS				
	16 at 48°	24 at 48°	36 at 48°	48	60
1:20	+	+++	+++	++++	++++
1:40	+	++	++	++	+++
1:80	?	+	+	+	++
1:160	–	?	+	+	+
1:320	–	–	–	–	–
Saline	–	–	–	–	–
Normal serum 1:20	–	–	–	–	–

+ to ++++ = degrees of agglutination visible macroscopically, ? = questionable agglutination, – = negative.

temperature after the proper time interval had elapsed. Table 1 shows the degree of agglutination apparent at various times during the incubation period of a typical test.

In the experiment tabulated in table 1, the rack was removed from the incubator at the end of 36 hours and held at room temperature during the remainder of the 60 hours. It will be noted that the final titer did not increase after 36 hours, but that the intensity of the agglutination showed some improvement. In a parallel experiment, the rack was removed at the end of 18 hours incubation and the final results were identical with those in table 1 at the end of 60 hours. This verifies the fact that once union between antigen and antibody has occurred, it is only a matter

of sufficient time elapsing before the results are visible, and the temperature of incubation after such a union is of little consequence. With a view to standardizing the technic, all incubations referred to in this report were done at 46 to 48°C. for 36 hours, followed by 24 hours at room temperature.

Heat-labile and heat-stable agglutinogens

Craigie and Wishart (1934) have reported the presence of at least two agglutinogens in a strain of vaccinia elementary bodies. The more labile L agglutinogen had its agglutinability and its ability to absorb agglutinin impaired or destroyed by exposure to a temperature as low as 56°C., while the S agglutinogen was stable at temperatures up to 95°C. Bedson (1933) showed that steamed formalinized psittacosis virus lost none of its immunizing powers.

In view of the interesting findings in regard to the agglutinins produced by unaltered vaccinia elementary bodies, it was considered of value to determine whether or not the elementary bodies of psittacosis showed any such antigenic complexity.

A preliminary experiment showed that a suspension of freshly prepared L.C.L. bodies, after being heated for 1 hour at a temperature of from 70 to 75°C., was still agglutinated by a known antiserum, but not by a normal guinea pig serum. This observation called for further investigation.

A suspension of freshly washed and concentrated elementary bodies was heated at 70 to 75°C. for 1 hour and then cooled. 0.5 ml. of this suspension was added to the same amount of a 1 in 10 dilution of antiserum having a titer of 1 in 160. This antiserum was produced in guinea pigs by the inoculation of freshly prepared virus. The mixture was incubated in the usual manner and showed a very good + + + agglutination at the end of 60 hours. The tube was then centrifuged at 3,000 R.P.M. in the ordinary horizontal centrifuge for 15 minutes, and the clear supernatant fluid removed. The sediment, when stained by the Castaneda method, showed clumps of elementary bodies obviously agglutinated by the antiserum. Saline and normal

serum controls were entirely negative when treated by the same procedure.

The absorbed serum was then set up with both freshly prepared elementary bodies, and with elementary bodies which had been heated at 70 to 75°C. for 1 hour. The mixtures were incubated in the usual manner. At the end of 60 hours, the heated elementary bodies showed no signs of agglutination, while the unheated material gave a definite ++ agglutination. Saline and normal serum controls were again negative, and agglutination was once more verified by stained smears. These findings were verified by further experiments of the same type, but due to the difficulty of obtaining large amounts of elementary bodies in the necessary state of purification, it was not possible to determine more accurately the temperature range at which the heat-labile component was destroyed.

On the basis of the above results, one may conclude that the elementary body suspensions, as prepared for purposes of immunization, contained at least two components, one destroyed at 70 to 75°C. for 1 hour and the other resisting the same treatment. These agglutinogens produced their specific agglutinins in the sera of inoculated guinea pigs, and these agglutinins were demonstrable by the usual absorption methods. It thus appears that the psittacosis elementary bodies are complex antigenically.

Cross-relationship with vaccinia agglutinogens

In view of the fact that both psittacosis and vaccinia elementary bodies possessed an antigenic complexity, it was considered of interest to determine whether or not one of these antigenic components might be common to both viruses.

A suspension of vaccinia elementary bodies was prepared in the same manner as the L.C.L. body suspensions were made. The vaccinia virus was obtained from a strain of egg membrane virus furnished in 1935 through the courtesy of Dr. E. W. Goodpasture of Vanderbilt University, and carried since that time by routine chorio-allantoic membrane passage. This virus has

gradually lost its invasive qualities for the skin of the normal rabbit, and now produces few or no lesions after over 350 egg-membrane passages. The elementary body suspensions prepared from this virus had the same appearance as the psittacosis suspensions already described.

Antivaccinia serum was prepared by hyperimmunization of normal rabbits with calf vaccinia virus. The shaved rabbits were given 0.4 cc. each of a 1 in 100, 1 in 1,000, 1 in 3,000 and 1 in 10,000 dilution of the virus by the usual scarification technic. This was followed in 19 days by the inoculation of 1 ml. of a 1 in 10 dilution of the same virus intravenously and the latter dose was repeated after another 17 day interval. The serum was collected and stored without preservative by cardiac bleeding 6 days after the last inoculation.

TABLE 2

Cross agglutinations with psittacosis and vaccinia elementary bodies and antisera

ANTISERUM	HEATED PSITTACOSIS ELEMENTARY BODIES	UNHEATED PSITTACOSIS ELEMENTARY BODIES	HEATED VACCINIA ELEMENTARY BODIES	UNHEATED VACCINIA ELEMENTARY BODIES
Vaccinia.....	Negative 1:20	Negative 1:20	Positive 1:10	Positive 1:160
Psittacosis...	Positive 1:160	Positive 1:320	Negative 1:10	Positive 1:40

Preliminary experiments showed that the psittacosis antiserum was able to agglutinate vaccinia elementary bodies in a titer of 1 in 40 by the usual technic. The vaccinia antiserum prepared in rabbits gave a doubtful agglutination of psittacosis elementary bodies in a dilution of 1 in 10. These observations led to further experiments with both heated and unheated elementary bodies of both types. These suspensions were set up with both vaccinia and psittacosis antisera. Table 2 summarizes in composite form the principal results obtained by such investigations.

The data in table 2 represent the highest significant dilution of the materials used. All saline and normal serum controls were negative in the dilutions used.

It is rather difficult to draw clear conclusions from such findings. The antipsittacosis serum was able to agglutinate unheated vaccinia elementary bodies, but not heated material.

It must be emphasized that the elementary bodies of both viruses were obtained from chick material, but that the vaccinia antiserum was prepared in rabbits receiving calf material only. The results observed were not due to chick antibodies in the guinea pig sera, as will be proved in a later section. Apparently the cultivation of the viruses on the chorio-allantoic membrane had resulted in the presence of some common factor, which made it possible for the vaccinia elementary bodies to be agglutinated in low titer by antipsittacosis serum. The only possible conclusion to be drawn from these puzzling observations is that under the conditions of the experiments performed, some common antigenic factor was present in egg membrane viruses of psittacosis and vaccinia, perhaps a component acquired by the intracellular habitat. Due to the difficulty of obtaining large amounts of material, it was not possible to investigate this phenomenon as thoroughly as might be desired.

Control experiments

In order to evaluate accurately the above results, it was necessary to rule out the possibility that one or both of the viruses investigated had become contaminated with the other. The ease with which vaccinia virus can spread through animal quarters is well known, and since both viruses were being constantly incubated and transferred in close proximity, it was considered possible that through carelessness or accident, some mixture might have occurred. Accordingly, elementary body suspensions of both types were inoculated intraperitoneally into mice, the psittacosis in a dilution of 1 in 10 and the vaccinia undiluted. The mice receiving the psittacosis material died with positive findings in 2 and 4 days. The mice receiving the undiluted vaccinia suspension were anatomically negative for psittacosis when sacrificed 70 days after inoculation. These results were verified by another experiment of the same type, and it was therefore known that the vaccinia virus suspensions contained no psittacosis virus as a contaminant.

To rule out the possibility of the psittacosis virus having been contaminated with vaccinia virus, intradermal inoculations were

made with suspensions of both types in separate normal white rabbits. Although neither suspension gave a definite vaccinia lesion, due to the peculiarities of the vaccinia virus strain used, further inoculation with calf virus verified the fact that the psittacosis material contained no vaccinia virus, since the animal receiving the psittacosis virus gave a positive vaccinia reaction upon inoculation with the calf virus, while the other rabbit showed a definite vaccinoid reaction, indicating partial immunization by the previous inoculation. These results were likewise verified by further experiments of the same type.

Further evidence of the purity of the strains was supplied by the picture given in the chorio-allantoic membrane. The psittacosis virus, as already described (Lazarus and Meyer (1939a)), gave an easily recognizable lesion without discrete foci, with death of the embryo between the third and fourth days. On the other hand, the vaccinia virus gave occasional discrete lesions typical of this virus, and the membrane showed none of the edema characteristic of the psittacosis infections. The eggs infected with vaccinia virus consistently showed death of the embryo between the second and third day. Thus, through animal experiments and pathological pictures, it appears certain that no mixture of the two viruses was responsible for the results described above.

Although the tests for protein in the wash water of the psittacosis suspensions were negative after the second washing following tryptic digestion, it is a known fact that biological tests for the presence of protein are much more delicate than any known chemical test. Accordingly, it was considered necessary to rule out the possibility of the presence of anti-chick-protein antibodies in the sera used to agglutinate psittacosis elementary bodies of chick origin.

Normal chick protein was prepared by grinding chorio-allantoic membranes removed from uninoculated eggs. The ground material was suspended in buffered saline, extracted overnight in the ice chest, centrifuged and the supernatant fluid removed. This material gave a very heavy precipitate when tested with sulfosalycilic acid. Guinea pigs and rabbits were inoculated

with this material by intraperitoneal and intravenous injections. The material was antigenically active, as was shown by the induction of anaphylactic shock in one guinea pig. The sera from these animals were not able to agglutinate the washed elementary body suspensions of psittacosis virus. Tests were made after 1 injection and after 5 injections, but no positive agglutination resulted when the sera were set up with L.C.L. body suspensions. It therefore appears certain that the results observed when psittacosis elementary bodies were agglutinated by antiserum were not due to the presence of antibodies against chick protein. These anti-chick sera were also used as control material for the precipitin tests described below.

Agglutination of Proteus vulgaris

Lillie (1930) noted the resemblance of the L.C.L. bodies to the rickettsia group and proposed the name "*Rickettsia psittaci*" for the psittacosis inclusion bodies. Meyer (1935) pointed out that there is no evidence at hand that psittacosis is an insect-borne disease and that the name proposed by Lillie was therefore inappropriate.

In view of the microscopic resemblance of L.C.L. bodies to the rickettsia group, it was considered of interest to determine whether the antipsittacosis serum was able to agglutinate proteus strains, a phenomenon well known in connection with rickettsia infections. Accordingly, suspensions of various proteus strains were prepared and washed by the usual technic. These suspensions were set up against a strongly positive psittacosis antiserum and were incubated in the usual manner for bacterial agglutinations.

The results observed were of little positive significance. *P. vulgaris* OX 19 and OX 2 were not agglutinated by the sera used. *P. vulgaris* OX K and OX Muar were agglutinated in dilutions of 1 in 20, a titer of very doubtful significance.

The same strains were set up against an antivaccinia rabbit serum. All agglutinations were negative by the standard technic.

It appears, in the light of the above results, that neither the

psittacosis nor vaccinia elementary bodies bear any close serological relationship to the rickettsia group.

Agglutinins in other sera

The results reported above were obtained by the use of sera from guinea pigs immunized with suspensions of washed and concentrated elementary bodies. It was considered of value to verify these results by appropriate tests of sera from other sources. The author is indebted to Miss Bernice U. Eddie of the Hooper Foundation for her coöperation in supplying the materials used in the studies below.

The agglutination tests described below were performed by the standard method described above, and appropriate saline and normal serum controls were included in each experiment. For the sake of brevity, the results are recorded in table form.

Discussion

The results shown in table 3 bring to light some interesting data. That the agglutination reaction is not due to the presence of chick protein in the antigenic material is proved by the positive agglutinations observed in guinea pig 1453, monkey 709, monkey "leather collar," and human AM. None of the foregoing had received chick protein in any form. Normal human, monkey, rabbit and guinea pig sera were consistently negative in all the above tests.

It may likewise be observed that the agglutination titer in monkeys was never as satisfactory as the titer in guinea pigs and rabbits receiving the same type of material. This emphasizes the known fact that monkeys are not the most satisfactory animals to use for studies of psittacosis virus. It is likewise noteworthy that the mouse virus suspensions were not antigenically adequate, since it was observed early in these studies that it was almost impossible to free the virus from mouse-organ cells. In the process of preparing the antigenic material used above, much virus was undoubtedly discarded with the sediment of centrifugalized mouse material, while the same applied to a greatly lessened extent with guinea-pig and chick virus. This

once again emphasizes the fact that the virulence and hence the antigenicity of the material used runs parallel with the number of elementary bodies present, and lends further support to the belief that the L.C.L. bodies are actually the virus particles.

The type of agglutination observed in all tests conducted varied with the type of serum used. Rabbit serum consistently

TABLE 3

Agglutination tests performed with miscellaneous sera

SOURCE	IDENTIFICATION	TITER	REMARKS
Rabbit.........	4	+ 1:160	Formalinized and normal crude chick virus intermittently for 2 years
Rabbit.........	8	− 1:20	Mouse liver and spleen extract intermittently for 2 years
Rabbit.........	92	+ 1:160	Crude egg membrane virus weekly for 2½ months
Rabbit.........	94	+ 1:320	Tissue media virus (chick) plus normal horse serum for 2 months
Guinea pig.....	1,074	− 1:10	Mouse virus and guinea pig lung virus intermittently for 1½ years
Guinea pig.....	1,453	+ 1:160	Guinea pig lung virus for 3 months
Monkey........	47	+ 1:80	Crude egg membrane virus for 2 years
Monkey........	71	− 1:20	Partially purified mouse virus for 3 years
Monkey........	709	+ 1:80	Mouse liver and spleen suspension for 2 years
Monkey........	1,837	+ 1:80	Crude egg membrane virus for 6 months
Monkey........	L.C.*	+ 1:80	Guinea pig lung virus for 1½ years
Human.........	BE	− 1:4	Working with virus. Positive complement fixation
Human.........	AL	− 1:20	Working with virus. Positive complement fixation
Human.........	AM	+ 1:160	75 days after first symptoms of proved clinical case

* L.C. = Leather collar.

gave large flaky clumps, which tended to settle completely, leaving the supernatant fluid crystal clear in all but the highest dilutions. On the other hand, human, monkey and guinea pig sera gave a granular agglutination with clumps which were easily disrupted. They settled very slowly and left the supernatant fluid clear only rarely, and then in tubes having a concen-

tration of serum far greater than the end-point of the titration. These results were observed consistently.

The results with human sera in table 3 emphasize the fact that the complement-fixation reaction is more delicate than the agglutination test. Human sera BE and AL both gave positive complement fixation reactions but no agglutinins were demonstrable in the dilutions noted. Comparison with guinea pig results shows that the agglutination test would probably be positive too late to be of any value in clinical diagnosis, whereas the complement fixation test was positive for human serum AM shortly after the onset of the disease. It is regretted that earlier specimens of this serum were not available for agglutination tests.

SUMMARY

1. Agglutinins for psittacosis elementary bodies have been produced in guinea pigs by the inoculation of washed L.C.L. body suspensions.

2. The technic of the agglutination test and the influence of incubation time and temperature have been studied.

3. The presence of heat-labile and heat-stable agglutinogens in psittacosis elementary bodies has been demonstrated.

4. Control experiments have proved that the agglutination reaction was not due to the presence of foreign protein or of virus contamination.

5. Cross-agglutination reactions with vaccinia virus and vaccinia antiserum have given some indication of a common antigenic factor with psittacosis virus.

6. Agglutinins have been demonstrated in the sera of human, monkeys and guinea pigs which were immunized with virus from sources involving no contact with chick protein.

7. No significant agglutinin titer for *P. vulgaris* strains was demonstrable in antipsittacosis serum.

MERRILL "MASS FACTOR" IN AGGLUTINATION

Review

Merrill (1936) has discussed the conditions under which aggregation reactions may be expected to occur. This worker

pointed out that an analysis of the requirements of reactions *in vitro* indicates that we need not yet assume a special mechanism for the immunological reactions of viruses *in vitro.* Merrill points out that the lack of observable reactions is quite probably due to the fact that insufficient antigenic mass is present.

As Merrill emphasizes, the most important consideration in *in vitro* reactions is how much actual antigen, having regard both to the number of particles and the size of the particles, is present in the virus preparations available. A rough approximation to the available experimental results is given by the assumption that 0.001 mgm. of antigen per ml., irrespective of the particle size or molecular weight, is the minimal amount capable of producing a visible aggregation with an active immune serum. Over the whole range of antigenic particles, from relatively simple molecules through virus particles to bacteria and red blood cells, there is a consistent deviation from this approximation, the smaller antigens (molecules) reacting with somewhat smaller numbers per ml., while the larger particles (bacteria and red blood cells) must be present in considerably larger numbers. Merrill's table is reproduced in figure 3.

It will be noted in figure 3 that the theoretical threshold serological reaction curve is based on 2 points, the mass of both antigens being 0.001 mgm. per ml. The diazo dye precipitation reaction was experimentally determined, and that point linked with the point represented by 0.001 mgm. per ml. of red blood cells by the dotted line. This line then represents the theoretical threshold of positive serological reactions, assuming that a mass of 0.001 mgm. per ml. is required. Actually, as previously discussed, there is a consistent deviation from this approximation, and the solid line in figure 3 represents the threshold curve as connecting two experimentally determined points, those for the pneumococcus specific soluble substance and for red blood cells. Along this line may then be placed the theoretical points for the various materials mentioned. It will be noted that the experimental point for paratyphoid bacilli coincides closely with the theoretical, while the two points for vaccinia virus are well within the limits of experimental error.

It was considered of value to determine whether the psittacosis

particle might be placed on Merrill's diagram, since the materials were available. The methods and calculations discussed below are arrived at following the formulae of Merrill.

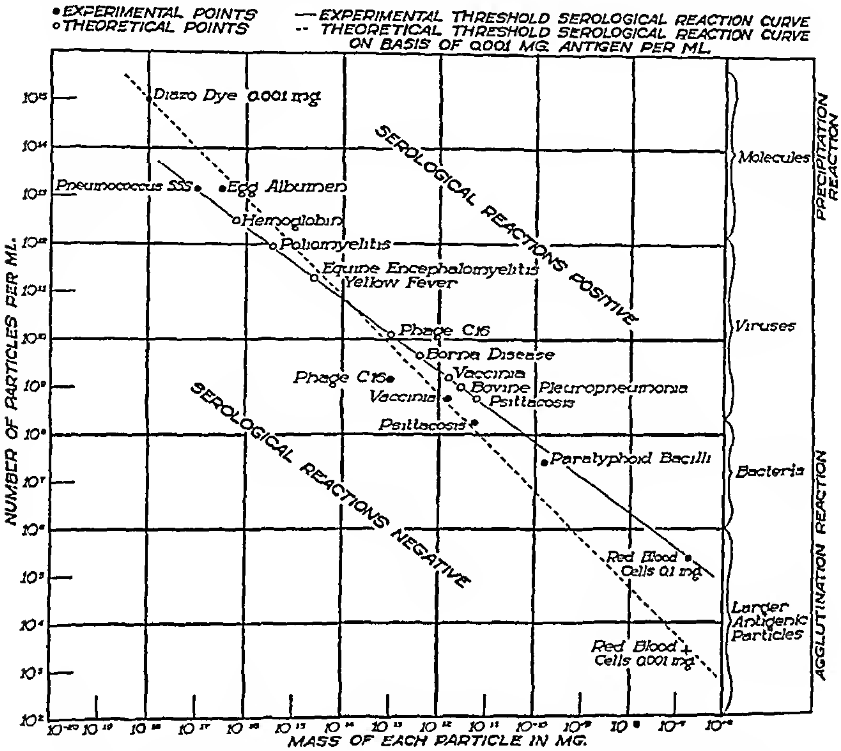

Fig. 3. The Mass Factor in Serological Reactions, after Merrill. Psittacosis Data Added

Experimental

The calculation of the mass of the psittacosis elementary body necessitated the assumption that the particle was a perfect sphere with a specific gravity of one. The error introduced by actual deviations from these assumptions could not affect the final result to any great extent, and it probably introduced no appreciable error to assume that the mass of the particle in milligrams was equal to the volume in cubic millimeters. Since the psittacosis elementary body has been shown to have a

diameter of 200 mμ to 300 mμ (Lazarus, Eddie and Meyer (1937)), a figure of 250 mμ was taken to give an approximate diameter. Applying this to the formula for the volume of a sphere, $\frac{4}{3}\pi r^3$, the approximate mass in milligrams of each L.C.L. body was found to be 7.7×10^{-12}. Applying this value to the curve represented by the heavy line in figure 3, it is seen that approximately 8×10^8 virus particles per ml. would be necessary to elicit a visible reaction. This figure is of course a theoretical one based on a curve with experimentally determined values at each end.

The actual number of elementary bodies necessary to give a visible reaction was determined and checked experimentally by using dilutions of a counted L.C.L. body suspension and observing the tube in which agglutination was just visible. The counts were made in the Petroff-Hausser chamber as already described (Lazarus and Meyer (1939a)) and the standard agglutination technic was followed throughout. A 1 in 10 dilution of an elementary body suspension containing 2.2×10^9 particles per ml. gave a definite positive agglutination test, while a 1 in 20 dilution of the same suspension gave no visible sedimentation, when set up with a known positive antiserum with the proper controls. It may, therefore, be stated that under the conditions of the test, a suspension containing 2.2×10^8 virus particles per ml. gave a positive agglutination test, while a suspension of 1.1×10^8 particles per ml. gave no visible clumping.

This value was then placed on the Merrill diagram and is represented by a solid dot. It will be noted that this point coincides closely with the theoretical line based on 0.001 mgm. antigen per ml. The mass of virus particles required for a positive reaction was then calculated and gave a figure of 0.0017 mgm. per ml. of psittacosis particles necessary before a positive agglutination reaction could be elicited under the conditions of the experiments. This value, it will be observed, agrees with Merrill's statement that the larger antigenic particles must be present in greater numbers than that represented by the theoretical 0.001 mgm. of antigen required.

On the basis of the Merrill theory, positive serologic reactions

have not been obtained with viruses because there has been an insufficient number of virus particles present, and, if the theory is correct, observable reactions will be obtained when the threshold virus concentration is reached. Until it has been shown that this factor has been taken into account, one is not justified in concluding that viruses differ fundamentally in their antigenic behavior *in vitro* from smaller or larger antigenic particles about which more is known.

The principles laid down by Merrill give a logical explanation for the unsatisfactory agglutination reactions with the sera of animals receiving psittacosis virus in low concentration, notably the partially purified mouse material. The theory likewise helps to explain the highly satisfactory results obtained with animals which had received large doses of washed and concentrated L.C.L. bodies. From these considerations it would seem probable that the problem of preparing immune sera against viruses in non-susceptible animals is primarily one of obtaining sufficient mass of antigenic agents. This question of obtaining sufficient mass of antigen might well be a formidable barrier in many virus diseases, but has been in part met in psittacosis.

SUMMARY

1. The studies of Merrill, regarding the relationship between the size of antigenic particles and the number of particles necessary to produce visible immunological reaction, have been applied to the virus of psittacosis.

2. The data obtained for psittacosis virus have conformed closely to the principles outlined by Merrill.

3. The application of Merrill's theory has shown that immunological reactions have not been obtained with some anti-psittacosis sera because insufficient concentrations of virus particles have been used as immunizing agents.

PRECIPITATION

Review

No investigations of the precipitin reaction in psittacosis have as yet been published. The early work of Craigie (1932) has

laid down a sound foundation for the studies of vaccinia precipitation. The important preliminary work which made the detailed investigation of the vaccinia flocculation reaction possible was done by Gordon (1925), Burgess, Craigie and Tulloch (1929) and Craigie and Tulloch (1931). The work of Craigie and Wishart and of Parker and Rivers has shown the presence of a soluble specific substance in the filtrate of vaccinia emulsions after all virus has been removed. The antibodies responsible for the precipitation reaction were the same as those causing agglutination of the washed elementary bodies. Absorption tests have shown that the antigen possesses both heat-labile and heat-stable fractions.

Precipitin reactions have been demonstrated for filtrates of myxoma virus (Rivers and Ward (1937)), using virus-free filtrates of emulsions prepared from infected skin. A specific precipitinogen was also demonstrated in virus-free serum of animals acutely ill as a result of extensive infection with myxoma virus.

Experimental technique

The technic followed in the precipitin tests with psittacosis material was similar to that outlined by Smadel and Wall (1937). These workers demonstrated a soluble precipitable substance in Seitz filtrates of vaccinia emulsions prepared from infected chorio-allantoic membranes. The supernatant fluid which resulted from the first angle centrifugalization of psittacosis-infected membranes was filtered through a Seitz EK pad, which had previously been prepared by the passage of 10 cc. of broth containing 1 ml. of normal rabbit or guinea pig serum. These filtrates were shown to be non-infectious for susceptible white mice. Serial dilutions of the clear serum-colored filtrates were prepared with buffered saline solution and mixed with equal volumes of diluted immune serum. Tubes and racks similar to those employed for agglutination tests were used and the mixtures were incubated overnight at 48°C.

The results obtained using the above technic were found to vary considerably, and no completely satisfactory demonstra-

tion of precipitation occurred under the conditions of the tests, although occasional positive results pointed toward the presence of antigenically active material. A typical test result is given in table 4.

These results are typical of those obtained throughout numerous attempts to demonstrate the presence of precipitinogens in the extracts of infected tissues. It will be noted that normal guinea pig serum gave a weakly positive reaction with high concentrations of chick protein, whether from normal or infected membranes. This presence of normal precipitins made it impossible to use the more concentrated antigenic material, and the necessary dilution resulted in very weak reactions.

TABLE 4

Soluble precipitable substances in filtrates of chick membranes infected with psittacosis virus

	PSITTACOSIS EXTRACT UNDILUTED	PSITTACOSIS EXTRACT 1-2	NORMAL EXTRACT 1-2
Antiserum 1:5	++	+	±
Antiserum 1:10	+	±	−
Anti-chick serum 1:5	++	±	+
Anti-chick serum 1:10	+	−	−
Normal serum 1:5	+	−	−
Normal serum 1:10	−	−	−
Saline	−	−	−

++, +, ±, − = degrees of precipitation.

Considerable difficulty was experienced with the so-called "anomalous flocculation" in tubes containing lower concentrations of serum. The same phenomenon has been reported by Craigie and Tulloch (1931) in their investigation of the variola-vaccinia flocculation reaction. It has been suggested by these workers that the anomalous non-specific flocculation appears to bear some resemblance to the "cloudy" reaction which sometimes interferes with the application of agglutination to the study of *Salmonella pullorum*. This subject is discussed in detail in the work of Valley and Casman (1930), and the reaction is apparently due to the presence of lipoid-protein complexes in the serum. The presence of this phenomenon in the psittacosis

precipitin tests led to much difficulty in the proper interpretation of results. Since it was not possible to extract the infected tissue by the methods suggested by Craigie and Tulloch (1931), due to the great danger of contaminating the equipment with active virus, it appeared that the precipitin method did not lend itself successfully to a study of this particular virus.

Attempts with positive rabbit and human sera yielded equally vague results, and the occasional significant findings obtained were not sufficiently clean-cut to make them of value. Membrane emulsions were filterable with difficulty through Seitz pads, even after removal of most of the particles by centrifugalization. Ultrafiltrates of such emulsions yielded results no more satisfactory than the Seitz filtrates. The presence of inhibitive substances in egg membrane filtrates has been noted by Smadel and Wall (1937), and these factors probably accounted for the variation in results. These workers also noted that filtrates of vaccinia membranes in certain passages did not contain any demonstrable serologically active substances. Apparently numerous uninvestigated factors are present in this type of material, and the low titer of precipitin sera prevents the demonstration of consistently clearcut results.

Craigie (1932) has demonstrated the antagonistic effect of complement on low titer precipitating sera. Inactivation of antipsittacosis serum in the above studies gave no noticeable improvement in results.

SUMMARY

1. The precipitinogens of filtrates of psittacosis-infected tissue have been investigated.
2. While results were not completely satisfactory, some indication of the presence of precipitins in low titer was obtained.
3. The factors contributing to the uncertain results have been investigated.

NEUTRALIZATION TESTS ON EGG MEMBRANE

Review

The use of the chorio-allantoic membrane of the developing egg for the titration of neutralizing antibodies in antiviral sera

has been advocated by Burnet and his co-workers (Burnet, Keogh and Lush (1937)). This group has devoted its efforts principally to the viruses of vaccinia, influenza, louping ill, infectious laryngotracheitis of fowls and rabbit myxomatosis. The method is of value only where well-defined lesions permit counting of pocks. The comprehensive monograph of Burnet (1936) summarizes the results obtained with these viruses.

In view of the lack of discrete lesions and because of the variability of the results in different egg membranes inoculated with the same material, it appeared unlikely that the psittacosis virus would lend itself to an accurate titration of neutralizing antibodies. This opinion was further strengthened by the fact that neutralization is difficult or impossible to demonstrate by any technic, even in sera of convalescent human cases (unpublished data). Nevertheless, an attempt was made to titrate serum-virus mixtures on the egg membrane.

Results

Table 5 gives the result of one such experiment. The serum virus- mixtures were held at room temperature for 1 hour before inoculation into 10 day eggs, in order that union of antigen and antibody might occur; 0.1 ml. of the serum-virus mixture was inoculated into each egg. The serum used was from a laboratory worker with a positive complement fixation reaction for psittacosis, and with some evidence of protection in neutralization tests performed in mice.

It will be immediately observed in the above experiment that observation of the time of death of the embryo is not a satisfactory method for determining the presence of neutralization of psittacosis virus, unless larger numbers of eggs and statistical methods are used. The absence of discrete lesions on the membrane makes it doubtful that this method will be of value in this application. Further experiments with immune guinea pig serum gave no indication of neutralization and the method was abandoned as unsatisfactory.

The reasons for failure of the method are apparent. It has already been shown in earlier sections that a relatively massive dose is necessary to infect the egg membrane. It is likewise

a known fact that neutralizing antibodies against this virus are present in exceedingly minute amount, if at all. If the amount of virus mixed with the serum under test were reduced sufficiently to allow the neutralizing antibodies to exert any influence, the amount of virus present, even in normal controls, would be too small to infect the egg. If the amount of virus were increased so as to be sufficient for egg membrane infection, the feeble action of the neutralizing antibodies could not be demonstrated.

TABLE 5

Neutralization of psittacosis virus as tested on the chorio-allantoic membrane

0.1 ml. immune serum	Dead 22 hours (trauma)	Hatched, 11 days	Hatched, 11 days
0.1 ml. of 1:100 virus	Dead 7 days, +	Dead 5 days, +	0
0.1 ml. of 1:1,000 virus	Dead 7 days, +	Dead 4 days, +	0
0.1 ml. 1:100 virus in normal serum	Dead 7 days, +	Dead 5 days, +	0
0.1 ml. 1:1,000 virus in normal serum	Dead 4 days, +	Dead 6 days, +	0
0.1 ml. 1:100 virus in immune serum	Dead 7 days, +	Dead 5 days, +	Dead 5 days, +
0.1 ml. 1:1,000 virus in immune serum	Dead 7 days, +	Dead 6 days, +	Dead 7 days, +

+ = positive for psittacosis, anatomically and by Castaneda stain, 0 = not done.

SUMMARY

1. Neutralizing antibodies could not be demonstrated by egg membrane inoculation, and the method was abandoned as unsatisfactory.
2. The reasons for failure of the method have been discussed.

Discussion of serological data

The foregoing sections have pointed out the close resemblance between the serological reactions of the elementary bodies of psittacosis and those of ordinary bacteria. The L.C.L. bodies have been shown to be good agglutinogens, although high titers in antisera could not be produced; they revealed antigenic complexity; some suggestion of antigenic relationship with vaccinia elementary bodies has been obtained; they followed closely the

general principles laid down by Merrill concerning the mass of antigen necessary to produce antibodies and to show visible *in vitro* reactions; they proved poor agents for the production of precipitins; and, finally, in the reacting sera, neutralizing antibodies could not be satisfactorily demonstrated. These data all yield further evidence to support the view that the etiologic agent of this disease is a minute microörganism having much in common with ordinary bacteria, and requiring an intracellular environment for multiplication and survival. The necessity for living tissue probably indicates that this agent is not equipped with the necessary enzyme systems which would allow independent existence.

It was regretted that the investigations outlined could not be carried out in greater detail. Owing to the difficulty of securing large quantities of virus, some basic information could not be obtained and other data were relatively incomplete. The production of 5 ml. of washed elementary bodies required approximately 60 eggs and several days' operations, and it is obvious that large quantities of material in a pure state could not always be guaranteed. The seasonal drop in the fertility of eggs also caused delays in the work. To these factors must be added the extreme caution needed in manipulating material with such infectious qualities. These points may help to explain the incomplete data and the omission of certain procedures.

GENERAL SUMMARY AND CONCLUSIONS

1. The virus of psittacosis has been propagated on the chorio-allantoic membrane of the developing egg for more than 425 consecutive bacteria-free passages without loss of infectiousness for susceptible white mice. The material obtained has been used for a wide variety of experimental studies.

2. Crude suspensions of the virus have been further purified and concentrated by means of tryptic digestion and differential centrifugalization. Standardization of the purified suspensions was accomplished by turbidity and by direct count of the elementary bodies.

3. Centrifugalization of the virus has resulted in concentrat-

ing the elementary bodies, but the supernatant fluid could not be entirely freed from infective particles.

4. Filtration experiments have demonstrated that the virus seldom passed through Berkefeld V and N, Chamberland L3 or Seitz EK filters. Highly successful filtration was obtained by using graded collodion membranes of the Elford type.

5. Measurements of the size of the infective particle by ultrafiltration have shown that the psittacosis virus is either the elementary body, an invisible particle not separable from the elementary body, or an invisible particle of the same size as the elementary body. The first theory seems most probable.

6. The elementary bodies have been shown to be good antigenic agents and to be antigenically complex. Satisfactory and reproducible agglutination reactions have been demonstrated. Some indication of positive precipitin tests has been obtained.

7. The mass of antigen needed for immunization and serologic reactions has been determined and the results accord well with the "mass factor" theories of Merrill.

8. Neutralization of the virus and demonstration of this reaction on the egg membrane were shown to be unsatisfactory for reasons demonstrated.

9. The virus of psittacosis, as represented by the elementary bodies, acts in all respects as a small microörganism requiring an intracellular habitat for multiplication and survival.

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A COMPARISON OF HYDROGEN PRODUCTION FROM SUGARS AND FORMIC ACID BY NORMAL AND VARIANT STRAINS OF ESCHERICHIA COLI[1]

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It is common knowledge that the gases formed by members of the colon-typhoid group of bacteria consist of mixtures of hydrogen and carbon dioxide. Pakes and Jollyman (1901) showed that pure cultures of organisms of this group produced mixtures of hydrogen and carbon dioxide from formic acid. Since they also produced these gases from glucose, Pakes and Jollyman concluded that the gas from glucose came from intermediately-formed formic acid. Harden (1901) showed that the most striking difference in the fermentation reactions of *Escherichia coli* and *Eberthella typhosa* is that the latter is unable to decompose formic acid to hydrogen and carbon dioxide. He made the further observation that increased pressure decreased the production of hydrogen and carbon dioxide and increased the amount of formic acid in the fermentation of glucose by *Escherichia coli.* The latter observation suggested the reversibility of the reaction, later demonstrated by Woods (1936).

It has generally been believed by most investigators of fermentations by the colon-typhoid group that the hydrogen formed during fermentation comes from intermediate formic acid. Most fermentation schemes, therefore, (e.g. Kluyver, 1931), designate formic acid as the precursor of the hydrogen formed.

However, Stephenson and Stickland (1932) and Stephenson

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(1937) present evidence that hydrogen may be produced by *Escherichia coli* from glucose and other carbohydrates, otherwise than from preliminarily-formed formic acid. Tasman and Pot (1935) could not confirm these findings.

Quastel and Whetham (1925), using the Thunberg methylene-blue technique, showed that *Escherichia coli*, which produces hydrogen and carbon dioxide from glucose, is able to reduce methylene blue in the presence of formic acid. Early studies in this investigation showed that certain strains of *Escherichia coli*, which had lost the ability to produce gas from sugars and sodium formate, had also lost the ability to reduce methylene blue with formic acid. This observation supported the suggestion that formic acid is the precursor of hydrogen and carbon dioxide in the fermentation of glucose by *Escherichia coli*.

However, Stickland (1929) showed that *Eberthella typhosa* which does not produce gas from glucose, is able to reduce methylene blue in the presence of formic acid. Consequently, factors other than the activation of formic acid must be operative in order that carbon dioxide and hydrogen be produced.

Since the work of Quastel, various investigators have studied the enzymes of bacteria of the colon-typhoid group which liberate gases from sugars and formic acid. Quastel (1925) described one of these as formic dehydrogenase, catalysing the reaction $HCOOH + R \rightleftharpoons RH_2 + CO_2$ where R is methylene blue or some other hydrogen acceptor. Another enzyme, hydrogenase, was studied by Tauss and Donath (1930) and Stephenson and Stickland (1931). Green and Stickland (1934) have shown that hydrogenase catalyses the reaction $H_2 \rightleftharpoons 2H^+ + 2(e)$ in a perfectly reversible manner.

Stephenson and Stickland (1932) have given the name "formic hydrogenlyase" to an enzyme which liberates hydrogen and carbon dioxide from formic acid, catalysing the reaction $HCOOH \rightleftharpoons H_2 + CO_2$. They are of the opinion that formic hydrogenlyase cannot be regarded as a combination of formic dehydrogenase and hydrogenase. Furthermore, they present evidence that hydrogenlyases other than formic hydrogenlyase, in particular glucose hydrogenlyase, exist.

From the work of Dienert (1900) and Karström (1938) it appears that the enzymes concerned in the fermentation of carbohydrates by bacteria may be of two types. Enzymes of the first type, called adaptive enzymes by Karström, appear in active form only when suitable substrates are present in the medium on which the organisms are grown. Enzymes of the second type, constitutive enzymes, are present independently of the substrates upon which they act.

Stephenson and Stickland (1933) have shown that formic hydrogenlyase can be classified as an adaptive enzyme. Organisms grown in broth alone do not produce gas from formic acid. However, the addition of sodium formate or glucose to growing cultures leads to the production of formic hydrogenlyase. The production of this enzyme does not occur in response to natural selection, nor is it necessarily accompanied by any considerable cell multiplication. This work is of special interest in view of the fact that some of the data here presented show that enzymes can be permanently or temporarily lost, and that the loss involved is a different phenomenon from the absence of an adaptive enzyme that may be regenerated by the addition of a suitable substrate.

The basis of this investigation is a comparison of the enzymes of normal *Escherichia coli*, with those of anaerogenic variants, in an effort to determine the nature of the enzymes concerned in the production of hydrogen from sugars and formic acid.

Numerous investigators have reported the occurrence of such anomalous strains. Neisser (1906) described *Bacillus coli-mutabile*, an organism that did not produce acid from lactose but consistently produced variants able to ferment that sugar. Penfold (1912) reported strains of *Eberthella typhosa* that failed to ferment dulcitol but gave rise to dulcitol-fermenting strains. Revis (1912) reported that strains of *Escherichia coli*, trained to tolerate malachite green, lost the ability to form gas on sugars. Penfold (1911) observed the same phenomenon with organisms grown with chloracetic acid. Similar phenomena have been reported by other investigators.

EXPERIMENTAL

The production of biochemical variants by a strain of Escherichia coli

Several years ago a strain of *Escherichia coli* (*communis*), from the collection at the University of Minnesota, was cultivated for use in testing germicides. Several transplants were made, and these were subcultured serially on nutrient broth containing Armour's peptone. The cultures were regularly plated and checked on glucose, maltose, lactose, sucrose, and mannitol broths. It was soon observed that certain cultures no longer produced gas on carbohydrates. Repeated plating of these cultures yielded a number of strains that behaved normally and a number that failed to produce gas on carbohydrate. Several of these variants were selected for further study. On repeated subculturing and plating, some reverted to gas production; others remained stable. Subculturing on sodium formate or carbohydrate broth favored reversion to gas production; however, some variants remained stable in spite of cultivation on carbohydrate medium.

All variants which failed to produce gas in sodium formate broth also failed to do so as from the usual carbohydrates. However, when variants reverted to gas production in sodium formate, they were then also able to produce gas from glucose and other carbohydrates. This point was checked repeatedly in view of the opinion expressed by Stephenson and Stickland (1933) and Stephenson (1937) that hydrogen may be produced from glucose without the intermediate production of formic acid, and that the enzymes responsible for the production of hydrogen from glucose and formic acid are distinct and separate. All cultures that produced gas from glucose also produced gas from sodium formate, and vice versa.

The colonies that appeared on plating were smooth, rough or intermediate in form. A large number of representative colonies were picked and tested for gas production. However, there was no correlation between roughness or smoothness of colony and ability to produce gas on carbohydrate broths. This was further confirmed by plating on deep agar.

Although most of the variants differed from the normal organism only in not being able to produce gas, we did encounter some variants that had lost the power to produce acid as well as gas from maltose. Cultivation of these on sugar broths favored reversion; however, stable strains were obtained which remained unable to produce acid on maltose even after serial transfer on maltose broth for several months.

Methods employed in the study of the enzymes of normal Escherichia coli and its variants

In this investigation, the enzymes of these organisms have been studied by the Thunberg methylene-blue technique as used by Quastel, or a modification of it; by measurement of pH changes in growing cultures to which various substrates were added; and by measurements of gas evolution with Warburg manometers.

Methylene blue technique. The organisms were grown regularly on nutrient broth containing beef extract, peptone and water. Carbohydrate broths contained in addition 0.5 per cent of stock sugar and Andrade's indicator. The medium used for the production of mass cultures was buffered by the addition of 0.5 or 1 per cent of disodium phosphate. All organisms used in enzyme studies were transferred daily for several days on broth before inoculation into the mass culture flask. Organisms were regularly checked on routine media.

The sugar solutions were sterilized by filtration through Berkefeld or Seitz filters, and added in appropriate amounts to the mass culture medium. Following inoculation, the cultures were incubated twenty-four to forty-eight hours at 38°C. Mass cultures were repeatedly centrifuged and resuspended in saline or Ringer solutions. The organisms must be washed carefully because the presence of extraneous organic matter will influence the results of the experiments. Since, in these experiments, the organisms catalyse reduction of methylene blue in the presence of specific substrates, it is necessary that other substances present in the culture which can act as hydrogen donators be removed so far as possible. Organisms such as *Aerobacter aerogenes* secrete capsular material which is difficult to remove by washing,

and frequently there is enough of this extraneous material in the culture to mask the reaction of the substrate.

Morphological variants which produce a firm pellicle or grow in granular form are difficult to suspend and reactions of such cultures are slow on all substrates. Except in the earlier experiments, suspensions were standardized by compacting in calibrated centrifuge cups, and resuspending the sediment in twenty times its volume of saline.

The earlier experiments were performed in tubes of the Thunberg type, in each of which were placed the suspension of organisms, a buffer, an oxidation-reduction indicator such as methylene blue, and the substrate to be tested. The tubes were evacuated, filled with oxygen-free nitrogen, and placed in a water bath. The time for decolorization of the indicator was determined by visual observation.

In the later experiments, a modification of the Thunberg technique was used. The test tubes were equipped with side arms containing soft rubber stoppers, such as are used on vaccine bottles. Each tube was closed with a two-hole rubber stopper providing an inlet and an outlet for the gas (oxygen-free nitrogen saturated with water), which was bubbled through the liquid in the tube. Oxygen was removed from the nitrogen by passage over hot copper gauze in a Kendall (1931) apparatus. The tubes were kept in a water bath at a definite temperature, usually 30°C. After the removal of entrapped air and the attainment of the proper temperature within the tube, the suspension of organisms was injected by means of a hypodermic syringe through the soft rubber stopper in the side arm. The decolorization of methylene blue was followed by visual comparison with control tubes, left open to the atmosphere, from which substrates had been omitted, and which contained varying amounts of methylene blue. Such comparisons showed the relative amounts of methylene blue that had been reduced, and facilitated the determination of the final end point.

These reduction experiments were controlled by setting up tubes containing organisms and methylene blue with no substrate. In order that the results be significant, it is necessary that the control tubes do not decolorize throughout the experi-

ment. There must be a significant difference in time of decolorization in the presence and in the absence of substrate. Using fresh suspensions, the methylene blue in the control tubes remained in the oxidized form for several hours, while with older suspensions, reduction might occur in a relatively short time.

Changes in pH of cultures. The same basic medium was employed in these studies as was used in the production of mass cultures. To this basic medium was added glucose, maltose or sodium formate solution that had been sterilized by filtration through Seitz or Berkefeld filters. After the addition of the carbohydrate, the medium was incubated for 48 hours at 37°C. to test for sterility. After inoculation, the cultures were again incubated for 48 hours. The pH of both the cultures and the uninoculated broth was determined at 30°C. by means of a glass electrode.

Warburg technique. The suspensions used were prepared as described above. Warburg manometers (Dixon, 1934) with nearly uniform cups of 20 ml. capacity were used. Into each cup 1 ml. of buffer, 1 ml. of 0.1M substrate, and 0.5 ml. of washed suspension of cells to be tested were introduced by means of pipettes; 0.3 ml. of a 30 per cent solution of potassium hydroxide was placed in the inner cup for the absorption of carbon dioxide. A stream of nitrogen purified by passage over hot copper was led through the manometer for ten minutes. The manometer was then placed in a constant temperature water bath at 30°C. and shaken with a 4 cm. stroke at a rate of 120 oscillations per minute. Ten minutes after placing the manometer in the thermostat, the manometric liquid was set at 0 and the stopcock closed. The evolution of gas was determined by measuring the rise of the manometer fluid in the open arm after adjusting the fluid in the closed arm to the zero point. Readings were therefore taken at constant volume. Manometers containing no substrate but the same volume of liquid were used as thermobarometric controls.

Reduction tests with normal and variant E. coli

The earlier experiments of this nature were performed with Thunberg tubes following the technique of Quastel (1932). Thunberg tubes contained the ingredients as listed in the tables.

The tubes were evacuated by a motor-driven air pump and placed in a water bath at 45°C. The time of decolorization was noted. In cases where decolorization proceeded slowly, the experiments were terminated before all the tubes were decolorized. In the first experiments reported, that amount of bacterial suspension was used which would give decolorization in approximately ten minutes with 0.01M sodium succinate.

Table 1 shows a comparison of the activation of sodium formate and sodium succinate by culture I, a normal *Escherichia coli*, with that of culture V, a variant unable to produce gas from sugars and sodium formate.

TABLE 1

Comparison of activation of sodium formate by cultures I and V

Culture	I	I	I	V	V	V
Tube number	1	2	3	4	5	6
1/5000 Methylene blue (ml.)	1	1	1	1	1	1
M/20 Sodium succinate (ml.)	0	0	1	0	0	1
M/15 Phosphate buffer, pH 7.5 (ml.)	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0	0	0.5	0	0
Suspension of organism (ml.)	0.5	0.5	0.5	0.4	0.4	0.4
Water (ml.)	2	2.5	1.5	2.1	2.6	1.6
Total volume (ml.)	5	5	5	5	5	5
Time of decolorization (min.)	3.5	>120	13.5	>120	>120	10.6

The two suspensions are comparable in their ability to reduce methylene blue with 0.01M sodium succinate. However, the variant culture was unable to reduce methylene blue in the presence of 0.01M sodium formate, whereas the normal organism did this in 3.5 minutes. The experiment was confirmed, using five tubes for each test.

In the normal fermentation of carbohydrates by *E. coli*, gas appears in a pH range of approximately 4.5 to 7.5. It was therefore considered desirable to investigate the activation of sodium formate by suspensions of normal and variant strains throughout this range.

Tables 2 and 3 illustrate the effect of pH on the time of decolorization with 0.01M sodium formate by cultures I and V. The suspension used in these experiments was not the same as that used in Experiment I; hence corresponding tubes do not show the same reduction time with 0.01M sodium succinate.

TABLE 2

Effect of pH on activation of sodium formate by culture I

Tube Number	1	2	3	4	5	6	7	8
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0	0
M/15 Phosphate buffer (ml.)	1	1	1	1	1	1	1	1
pH	7.5	6.9	6.5	5.9	5.2	4.4	7.2	7.2
M/20 Sodium succinate (ml.)	0	0	0	0	0	0	1	0
Suspension of organism (ml.)	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Water (ml.)	1.8	1.8	1.8	1.8	1.8	1.8	1.3	2.3
Total volume (ml.)	5	5	5	5	5	5	5	5
Time of decolorization (min.)	3	2.5	3	3	3.5	4	10	60

TABLE 3

Effect of pH on activation of sodium formate by culture V

Tube Number	1	2	3	4	5	6	7	8
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0	0
M/15 Phosphate buffer (ml.)	1	1	1	1	1	1	1	1
pH	7.5	6.9	6.5	5.9	5.2	4.4	7.2	7.2
M/20 Sodium succinate (ml.)	0	0	0	0	0	0	1	0
Suspension of organism (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Water (ml.)	2	2	2	2	2	2	1.5	2.5
Total volume (ml.)	5	5	5	5	5	5	5	5
Time of decolorization (min.)	60	70	>90	>90	>90	>90	10	64

It is evident that variant culture V was unable to activate sodium formate to reduce methylene blue throughout this range of pH. Decolorization of the control tube containing no substrate at pH 7.2 indicated that the cells had not been washed free of oxidizable matter.

The increase in time required for decolorization with increasing acidity, as shown in table 3, may be due to the effect of pH on the extraneous substances produced by the cells. Tube 8, a control with no substrate added, was reduced in a time intermediate between the times of reduction in tubes 1 and 2 containing sodium formate, the tubes most nearly corresponding in pH. The cells in this experiment were not fresh, as indicated by the reduction time for tube 8, a control.

Culture VII, a variant, and culture VIII, producing gas from carbohydrates and sodium formate, were isolated from the same

TABLE 4

Comparison of activation of sodium formate by cultures VII and VIII

Culture	VII	VII	VII	VIII	VIII	VIII
Tube number	1	2	3	4	5	6
1/5000 Methylene blue (ml.)	1	1	1	1	1	1
M/20 Sodium succinate (ml.)	0	0	1	0	0	1
M/10 Sodium formate (ml.)	0.5	0	0	0.5	0	0
M/15 Phosphate buffer, pH 7.5 (ml.)	1	1	1	1	1	1
Suspension of organism (ml.)	1.2	1.2	1.2	1.7	1.7	1.7
Water (ml.)	1.3	1.8	0.8	1.8	2.3	1.3
Total volume (ml.)	5	5	5	5	5	5
Time of decolorization (min.)	>120	>120	13	3.7	>120	8

source, a variant culture which reverted to gas production after continued cultivation on carbohydrate media. Since it was possible that gas production might depend on more than one factor, it was of interest to determine whether the property of activation of sodium formate was present in these cultures.

This experiment showed that culture VIII activated sodium formate strongly, whereas culture VII, from the same parent culture, did not activate sodium formate. Previous testing of the parent culture had shown that the ability to activate sodium formate was absent.

In the above experiments the mass cultures employed were grown on nutrient broth. In all experiments with variant or-

ganisms, the ability to activate sodium formate to reduce methylene blue had been found lacking, whereas this ability had always been found in the normal organism. The question then arose as to whether the enzyme which activates formate is an adaptive enzyme which is lacking in the variant because of absence of ingredients stimulating its formation in the medium employed in growing the mass cultures.

Yudkin (1932) and Stephenson and Stickland (1932) have shown that the enzymes concerned in gas production by *Escherichia coli* may be considered to be adaptive. It was therefore necessary to grow the normal and variant cultures on media which might stimulate the production of such enzymes.

In the next series of experiments, normal and variant organisms were tested for action on sodium formate and molecular hydrogen when grown on phosphate-buffered broth, and on phosphate broth containing glucose or maltose.

The modified methylene-blue technique described earlier was used. Suspensions of washed organisms were standardized by centrifuging in graduated tubes and made up to the desired volume, usually twenty times the volume of the organisms.

A considerable number of strains of normal and variant *Escherichia coli* were tested. Typical results are shown in table 5. The reduction experiments were terminated at sixty minutes; lack of decolorization of methylene blue at this time is indicated by $>60'$.

It is apparent from table 5 that culture II, normal *Escherichia coli*, can activate sodium formate and molecular hydrogen to reduce methylene blue regardless of the presence or absence of glucose or maltose in the medium used for growth. A considerable number of strains of *Escherichia coli* isolated from sewage were tested for their ability to reduce methylene blue with sodium formate and with molecular hydrogen. All possessed active enzymes, irrespective of the medium used for growth. Thus the enzymes concerned in the activation of hydrogen and sodium formate must, according to Karström, be considered constitutive. Cultures of anaerogenic variants which had reverted to gas production were tested for these enzymes (see

table 4). In every case organisms able to produce gas were found to possess hydrogenase and formic dehydrogenase.

The various types of anaerogenic variants found are shown in table 5. The activities of the enzymes, formic dehydrogenase and hydrogenase, varied considerably in different strains, but did not appear to be dependent upon the presence of glucose or maltose in the medium used for growth. Most of the variants

TABLE 5

The activation of sodium formate and molecular hydrogen by normal and variant Escherichia coli

Atmosphere	Nitrogen						Hydrogen		
Medium used for growth	b.	g.b.	m.b.	b.	g.b.	m.b.	b.	g.b.	m.b.
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1	1
M/15 Phosphate buffer, pH 6.9 (ml.)	5	5	5	5	5	5	5	5	5
M/10 Sodium formate (ml.)	2	2	2	0	0	0	0	0	0
Water (ml.)	0	0	0	2	2	2	2	2	2
Suspension of organisms (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total volume (ml.)	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
	Time of decolorization in minutes								
E. coli II	2	3.4	3	>60	>60	>60	6.2	6.2	4.2
Variant X	>60	>60	>60	>60	>60	>60	16	4	8
Variant XI	1.2	2.2	1.7	>60	>60	>60	>60	>60	>60
Variant XII	2.2	6.7	3.5	>60	>60	>60	3	4.5	3
Variant XIII	>60	>60	>60	>60	>60	>60	44	>60	54

b., phosphate broth; g.b., glucose phosphate broth; m.b., maltose phosphate broth.

tested were of the type of Variant X, that is, they were unable to activate formate. In only one case was an anaerogenic culture (Variant XII) found which was able to activate both hydrogen and formate, i.e., possessed both hydrogenase and formic dehydrogenase. It is apparent from the existence of this culture that some factor other than the presence of these enzymes is necessary for production of gas. Further evidence to this effect arises from the fact that a suspension of normal *Escherichia coli* grown in broth without proper substrate produces little or no

gas from glucose or sodium formate, whereas gas is produced at a rapid rate if sugars are present in the medium used for growth.

Stephenson and Stickland (1932) and Stephenson (1937) have concluded that formic hydrogenlyase, the enzyme concerned in the production of gas from sodium formate, is an enzyme separate and distinct from formic dehydrogenase and hydrogenase. This is in opposition to their earlier view (Stephenson and Stickland 1931) that formic dehydrogenase and hydrogenase together make up the enzyme producing gas from formic acid. Their conclusion was based in part on evidence that *Bacillus lactis-aerogenes* possessed no hydrogenase, but was able to produce gas from formic acid, i.e., possessed formic hydrogenlyase. In their earlier paper Stephenson and Stickland considered the presence of hydrogenase in *B. lactis-aerogenes* questionable since reduction with hydrogen in Thunberg tubes took place in 9½ minutes, whereas reduction occurred in 15½ minutes in the control. In their second paper, this question was reëxamined, and the conclusion reached that the enzyme was absent, but no conclusive data were given.

It seemed, therefore, desirable to test cultures of *Aerobacter* species for the presence of hydrogenase.

Hydrogenase in aerobacter species

Three strains of *Aerobacter aerogenes* and two strains of *Aerobacter cloaceae* were subcultured in broth containing 0.25 per cent sodium formate and 0.25 per cent glucose every three days for a month. Mass cultures were then grown in broth containing 1 per cent phosphate, 0.25 per cent sodium formate and 0.25 per cent glucose. The organisms were harvested at 36 hours and washed three times in 0.9 per cent saline. The suspensions were then tested for hydrogenase, using the modified methylene blue method. The results are shown in table 6.

Suspensions of *Aerobacter aerogenes* 401 were further tested for hydrogenase in solutions of varying pH, as shown in table 7.

It is clear that the suspension of *Aerobacter* species tested reduced methylene blue more rapidly in the presence of hydrogen than in the presence of nitrogen. In relatively concentrated

suspensions, the reducing matter associated with the cells masked the presence of hydrogenase. The effect of change in pH shows that more than one factor is operating in those tests where hydro-

TABLE 6

Tests for hydrogenase in aerobacter species

CULTURE	SUSPENSIONS OF ORGANISMS USED					
	1:20		1:40		1:100	
	Times of reduction of methylene blue in minutes in atmospheres of					
	H_2	N_2	H_2	N_2	H_2	N_2
Aerobacter aerogenes 401	2.0	18	2.7	51	10	>90
Aerobacter aerogenes 403	2.7	10.4	4.4	32	16	>90
Aerobacter aerogenes 404	2.3	10.8	7.1	>90		
Aerobacter cloacae 451	2.8	5.1	6	13	12	>90
Aerobacter cloacae 452	4	61				

Suspensions of organisms are from mass cultures grown for 36 hours at 37°C. on 1 per cent phosphate broth with 0.5 per cent glucose and 0.25 per cent sodium formate.

Each tube contained: 5 ml. M/15 phosphate buffer, pH 6.9, 0.5 ml. 1/5000 methylene blue, 0.5 ml. suspension of organisms.

Temperature of experiment: 30°C.

TABLE 7

The effect of pH and concentration of cells on hydrogenase in aerobacter aerogenes

pH	SUSPENSIONS OF ORGANISMS USED					
	1:20		1:40		1:100	
	Times of reduction of methylene blue in minutes in atmospheres of					
	H_2	N_2	H_2	N_2	H_2	N_2
6.3	3.2	20	4.5	63	18	>120
6.7	2.5	18.5	3	51	13.5	>120
7.1	1.8	17.5	2.5	53	10.5	>120
7.5	2	17	3	48	8.2	>120
7.8	3	12.5	3.2	47	8	>120

Suspensions from a mass culture grown for 36 hours at 37°C. on 1 per cent phosphate broth with 0.5 per cent glucose and 0.25 per cent sodium formate.

Each tube contained: 5 ml. M/15 phosphate buffer, 0.5 ml. 1/5000 methylene blue, 0.5 ml. suspension of organisms.

Temperature of experiment: 30°C.

gen was used. Hence it must be concluded that these *Aerobacter* species possess hydrogenase.

TABLE 8

Final pH values of media inoculated with normal and variant strains of E. coli

ORGANISM	REACTION ON GLUCOSE BROTH (Using Durham tubes)		REACTION ON MALTOSE BROTH (Using Durham tubes)		BROTH	GLUCOSE BROTH	MALTOSE BROTH	FORMATE BROTH
					pH	pH	pH	pH
Uninoculated					7.1			
E. coli II	A	G	A	G	7.2	4.7	5.0	8.1
Variant X	A	—	A	—	7.1	4.8	4.8	7.1
Variant XI	A	—	—	—	7.1	4.8	7.0	7.0
Variant XII	A	—	A	—	7.1	4.7	5.2	7.0
Variant XIII	A	—	—	—	7.2	5.4	7.0	7.1

TABLE 9

Hydrogen evolution by Escherichia coli from sodium formate, glucose and maltose

SUBSTRATE	MEDIUM USED FOR GROWTH	GAS EVOLVED IN MM³ AT (TIME IN MINUTES) 0	5	10	15	20	30	45	60	90
Formate	b.	0	−1	−1	−2	−2	−1	−1	0	1
	g.b.	0	7	16	24	33	52	80	109	165
	m.b.	0	53	111	145	187	255			
Glucose	b.	0	0	1	2	5	11	28	57	135
	g.b.	0	5	9	16	23	41	68	97	158
	m.b.	0	15	33	52	68	108	165	223	
Maltose	b.	0	−1	−1	0	2	6	19	35	76
	g.b.	0	4	8	13	17	28	52	86	159
	m.b.	0	26	57	101	153	225	294		

b., phosphate broth; g.b., glucose phosphate broth; m.b., maltose phosphate broth.

Warburg cups contains: 1 ml. pH 6.9 M/15 phosphate buffer, 1 ml. 0.1M substrate, 0.5 ml. suspension of organism.

Inner cup contains: 0.3 ml. 30 per cent potassium hydroxide.

Temperature of experiment: 30°C.

Changes in pH by normal and variant Escherichia coli

The pH values were determined in cultures of normal and variant *Escherichia coli* grown at 37°C. for 48 hours in 1 per cent

phosphate broth, and in phosphate broth containing 0.5 per cent glucose, 0.5 per cent maltose, or 0.5 per cent sodium formate.

It will be seen that the variant cultures producing no gas from sugars and formate are inert towards sodium formate, whereas the normal culture produces a pronounced alkalinity in formate broth. Furthermore, the variant cultures which, according to the Durham tube criterion, are unable to produce acid from maltose do not change the pH appreciably in the presence of maltose.

Hydrogen evolution by normal and variant Escherichia coli

Suspensions of normal and variant *Escherichia coli* were tested for production of hydrogen from formic acid, glucose and maltose in Warburg manometers.

The variant cultures tested for hydrogen production were negative in all cases, confirming their anaerogenic character indicated by the usual carbohydrate media. The results obtained with the normal strain of *Escherichia coli* are given in table 9.

The suspension grown on broth produced no hydrogen from sodium formate, but produced gas from glucose and maltose after a latent period. Suspensions grown in glucose or maltose broths produced gas at once from sodium formate, glucose or maltose.

DISCUSSION

It is highly probable that the hydrogen produced from sugars by *E. coli* comes from intermediately-formed formic acid. Variant strains of *E. coli* which fail to form gas from sugars are unable to produce gas from formate. Variant strains reverting to the aerogenic form, following cultivation on sugar broths, produce gas from formate as well as from sugars.

Anaerogenic variants of *E. coli* may or may not possess the enzymes hydrogenase and formic dehydrogenase in active form. However, on reversion to the aerogenic form, these enzymes appear in active form and their presence is independent of the presence of glucose or maltose in the medium used for growth. Hydrogenase and formic dehydrogenase have been found in all strains of *E. coli* capable of producing gas.

The determination of the presence of hydrogenase in *Aerobacter* species makes tenable the theory that the production of hydrogen and carbon dioxide from formic acid is the result of the combined action of the enzymes hydrogenase and formic dehydrogenase, and makes unnecessary the postulation of formic hydrogenlyase as an enzyme separate and distinct from, and not composed of hydrogenase and formic dehydrogenase.

Farkas, Farkas and Yudkin (1934), studying the decomposition of sodium formate by *Escherichia coli* in heavy water of varying deuterium content at pH 7, have shown that the hydrogen evolved by the decomposition of formate, with regard to its deuterium content, was in equilibrium with the water in the solution from which it was liberated. Analysis of the hydrogen evolved with regard to the three different molecular species H_2, HD and D_2 showed that these three constituents were present in their equilibrium concentration. In addition, the observation was made that if normal water was left in contact with hydrogen containing 30% D in the presence of *Escherichia coli*, a complete replacement of the deuterium gas by normal hydrogen occurred in a few hours.

They concluded that reactions of the type:

$$\mathrm{H\,COO\,Na} + \mathrm{D_2O} = \mathrm{H\,COO\,D} + \mathrm{Na\,O\,D}$$
$$\mathrm{H\,COO\,D} = \mathrm{H\,D} + \mathrm{CO_2}$$

could not express the decomposition of formate. Since only the hydrogen atom in the acid radicle can be replaced by D, D_2 can not be formed according to this reaction.

Krebs (1937), on the basis of the experiments performed by Farkas, Farkas and Yudkin, suggested that the mechanism of decomposition of formate can be represented by the following equation:

$$\mathrm{H_2O} + \mathrm{HCOO^-} = \mathrm{H_2} + \mathrm{H\,CO_3^-}$$

where the formate is oxidized by the water, accompanied by a liberation of hydrogen. According to this view, the hydrogen evolved is derived from the water of the medium, and is therefore in equilibrium with the water in respect to deuterium content in the experiments quoted above.

Borsook (1935) studied the reaction:

$$\text{pyruvate}^- + \text{formate}^- + H_2O = \text{lactate}^- + HCO_3^-$$

in the presence of toluene-treated suspensions of E. coli. The components of this reaction may be written:

(a) $\text{formate}^- + H_2O = HCO_3^- + 2H^+ + 2e, \ E_0' = -.41$

(b) $\text{pyruvate}^- + 2H^+ + 2e = \text{lactate}^-, \ E_0' = -.18$

It was discovered that pyruvate and formate do not react at a significant rate in the presence of toluene-treated organisms unless a dye is present whose E_0' is intermediate between that of the lactate-pyruvate and formate-bicarbonate systems. The explanation given for the necessity of an intermediary dye system is that in the toluene-treated organisms, electron conduction cannot occur between the lactate-pyruvate and formate-bicarbonate enzymes.

Borsook's theory of complete and incomplete enzyme centers may well be applied to the decomposition of formate by *E. coli.* A suspension of *E. coli* grown in broth alone is unable to produce gas from formate, and is also unable to reverse the reaction and synthesize formate from hydrogen and carbon dioxide (see Woods 1936), although the enzymes, formic dehydrogenase and hydrogenase, can be shown to be present. Our work shows that cells grown in broth containing sugars are able to produce gas from formate. According to Stephenson, this is also true of cells grown in broth containing formate. Because of this phenomenon the enzyme liberating gas from formate is said to be "adaptive." It is possible that in the suspension of *E. coli* grown in broth alone, some factor may be lacking so that electron conduction between hydrogenase and formic dehydrogenase cannot occur. On the other hand, cells grown on formate or carbohydrate broth produce gas from formate because this missing factor is present, and hence electron conduction can occur.

When we consider the synthesis of formate:

$$H_2 + HCO_3^- = H_2O + HCOO^-$$

it is evident that the enzyme system must be able to activate molecular hydrogen as well as bicarbonate. Since such a system

must therefore possess the combined characteristics of the two enzymes, hydrogenase and formic dehydrogenase, it seems reasonable to believe that the production of hydrogen and carbon dioxide from formate is the result of the combined action of these same enzymes.

The two components of the reaction:

$$H_2O + HCOO^- = H_2 + HCO_3^-$$

may be expressed as:

$$\text{(a)} \quad H_2O + HCOO^- = HCO_3^- + 2H^+ + 2e$$
$$\text{(b)} \quad 2H^+ + 2e = H_2$$

According to this scheme, the nature of the hydrogen evolved would necessarily be determined by the nature of the water. This is in agreement with the results of Farkas, Farkas and Yudkin in their experiments with heavy water.

From experiment IX it is apparent that resting cells which have been grown in plain broth are not able to regenerate the enzyme hydrogenlyase in the presence of formate, but are able to do so in the presence of glucose or maltose. According to Stephenson, organisms which have been grown in plain broth are able to produce the enzyme hydrogenlyase if they are subsequently cultured on broth containing formate. The fact that formate can bring about the regeneration of this enzyme in the one case where organisms are grown in its presence, and not in the other, indicates that the regeneration is controlled by factors other than the mere presence of formate. This question is being studied further, and will be discussed in greater detail in a later publication.

SUMMARY

Variant strains of *Escherichia coli* were isolated which produced no gas from formate and carbohydrate broths. Culturing in formate and carbohydrate broths favored reversion to the aerogenic form. In all cases, variants reverting to gas production produced gas from formate as well as from glucose and other carbohydrates.

No correlation was found between roughness and smoothness of colony and ability to produce gas.

A further variation, loss of power to form acid from maltose, occurred in some anaerogenic strains.

Anaerogenic variants have been found that possess both hydrogenase and formic dehydrogenase, others have been found that possess only one of these enzymes, and still others have neither of them. However, the enzymes are always present in cultures which have reverted to the aerogenic form. All normal strains of *Escherichia coli* were found to possess these enzymes in active form, irrespective of the presence or absence of glucose or maltose in the medium used for growth.

Three strains of *Aerobacter aerogenes* and two strains of *Aerobacter cloacae* were shown to possess the enzyme hydrogenase, although the presence of this enzyme may be masked by reducing systems associated with the bacterial cells.

The determination of pH changes in growing cultures showed that the anaerogenic variants were inert towards formate, and that the maltose variants produced no significant amount of acid from maltose.

All anaerogenic variants failed to produce hydrogen from maltose, glucose and formate when tested in Warburg manometers. Normal *Escherichia coli* grown in phosphate broth produced no gas from formate but did produce gas from maltose and glucose after a latent period. Suspensions of *Escherichia coli* grown in glucose or maltose broth produced hydrogen from formate, glucose or maltose at a rapid rate.

The results of our investigation indicate that the hydrogen produced from glucose by *Escherichia coli* comes from formic acid, which is an intermediate product in the fermentation of sugar, and that formic dehydrogenase and hydrogenase are constituents of the enzyme liberating gas from formic acid.

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INHIBITION OF PROTEINASES OF CERTAIN CLOSTRIDIA BY SERUM

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Some members of the gas-gangrene group, such as *Clostridium histolyticum*, secrete proteolytic enzymes which cause much tissue damage *in vivo*. However, certain non-pathogenic Clostridia, such as *Clostridium sporogenes*, produce proteolytic enzymes which are as active as those of *Clostridium histolyticum* in the hydrolysis of gelatin or casein, but which are relatively impotent *in vivo*. In regard to the pH optima and activation behavior, no differences have been found between the proteinases of the pathogenic and the non-pathogenic Clostridia (Weil, Kocholaty and Smith, 1939).

Pozerski and Blanc (1920) reported that the proteinase of *C. sporogenes* was inhibited by normal horse serum, while that of *C. histolyticum* was not. This suggested that absence of inhibition by normal serum of the exocellular proteinases of this group of organisms might be correlated with the pathogenicity of the strain producing the proteinase.

Evidence in support of this possibility appeared when, during the prosecution of this study, Grassmann (1938) reported the inhibition of the proteinase of *Clostridium botulinum* by normal horse serum, but could find no inhibition of the enzymes of *Clostridium welchii*. The enzymes of the latter organism, however, were inhibited by immune serum.

MATERIALS AND METHOD

The organisms used in this study, with the exception of the strain of *C. histolyticum*, were obtained from the American Type

Culture Collection. The smooth strains of *C. histolyticum* were obtained from Dr. Weinberg, of the Pasteur Institute, Paris; the rough strains were obtained by colony selection (Hoogerheide, 1937). They were grown in beef-heart infusion broth at 37°C. for 24 to 48 hours. After incubation, the cultures were centrifuged and the supernatant fluids passed through Seitz filters. These filtrates were used as the enzyme solutions.

The method used for the determination of proteolytic activity was a modification of the method of Haines (1932, 1933). The activity of the enzyme solution was calculated from the rate of gelatin liquefaction, measured in Ostwald viscosity tubes. In the comparison of the inhibiting action of certain serum fractions on bacterial proteinases with that on trypsin, Bactotrypsin was diluted until it liquefied gelatin at approximately the same rate as did the enzyme with which it was being compared.

PREPARATION OF GELATIN

Twenty-five grams of Coignet Gold Label gelatin were soaked in 150 ml. of water for three or four hours and then warmed gently until dissolved. A solution of 2.5 grams of phenol in a small quantity of water was added to the gelatin as a preservative. The whole was adjusted to pH 7.0 with 0.1 N NaOH. After dilution to 250 ml. with water, 211 ml. of 0.1 M Na_2HPO_4 and 39 ml. of 0.1 M NaH_2PO_4 were added. The solution was allowed to stand at 37°C. overnight, during which time a precipitate of calcium phosphate formed, which was removed by centrifuging. As was pointed out by Haines (1933), the viscosity properties of gelatin change somewhat with repeated liquefaction and gelation. To avoid this, the stock solution was divided into smaller lots and stored in the refrigerator. For each series of determinations, a fresh tube was liquefied at 37°C.

DETERMINATION OF ENZYME ACTIVITY

Since, when a number of simultaneous determinations were being made, it was necessary to use non-uniform viscosity tubes, these were calibrated by determining the outflow time for distilled

water at 37°C. This factor (t_w = the outflow time in seconds for distilled water, using 5 ml. total volume) was used in calculating the results. Simple Ostwald viscometers were used throughout. Best results were obtained in a given series when the t_w values for the different viscometers were approximately equal, preferably between 40 and 70.

Previous to each experiment, the viscometers and the solutions to be used (gelatin and enzyme) were brought to constant temperature in the thermostat. For each determination, 3 ml. of the 5 per cent gelatin solution, a suitable quantity of the bacterial filtrate (usually 0.1 to 2.0 ml.), and sufficient water to make the total volume exactly 6 ml., were mixed, the time of mixing being noted to the nearest half minute. Five ml. of this mixture were measured into the viscometer. The falling time of the mixture (t) was then measured to the nearest half second, every four or five minutes during the course of the determination. For purposes of calculation, the time (T) at which the viscosity measurement (t) was made, was taken as the interval, in minutes to the nearest half minute, between the mixing time and the mean time of each measurement.

When serum, or serum fractions, were used as inhibiting agents, they were added to the enzyme solution two minutes before the addition of the gelatin. The length of this time interval was purely arbitrary. One-tenth ml. of serum, or a corresponding amount of serum fractions, was used in each experiment.

CALCULATION OF RESULTS

The experimentally observed outflow time (t, in seconds) is plotted against the square root of the elapsed time (T, in minutes). A straight line results, the slope of which is calculated from the relation

$$t_1 - t_2 = k(\sqrt{T_2} - \sqrt{T_1}) \qquad \text{(A)}$$

The magnitude of the constant, k, depends, of course, on the constant of the particular viscometer used. As is shown below, it is also directly proportional to the square root of the enzyme

quantity used in the determination. To correct for these factors, use is made of the equation,

$$\frac{100\,k}{t_w} = K\sqrt{V} \tag{B}$$

where t_w is the viscometer constant, and V is the volume in milliliters of the enzyme solutions used. The factor 100 is introduced merely so that the values for K, the enzyme activity, will in general be above unity.

The equations presented above are forms of Schütz's law (1885). Haines (1932, 1933) has shown that equation (A) holds for the liquefaction of gelatin by filtrates of a variety of microorganisms. It was found to hold also for the bacterial enzymes

TABLE 1

Influence of amount of enzyme on gelatin liquefaction

VOLUME OF FILTRATE	k	t_w	$\frac{k}{t_w}$	K
ml.				
2.0	4.89	55.6	0.088	6.20
1.5	4.35	57.3	0.076	6.23
1.0	2.41	40.8	0.059	5.90
0.75	2.41	45.5	0.053	6.16
0.50	1.92	49.4	0.043	6.08
0.25	1.24	42.9	0.029	5.94

used in this study. Haines carried out his work using a constant volume (2.5 ml.) of enzyme solution throughout. This procedure is justified when filtrates of moderate enzymatic activity are used. It is often necessary, however, to measure very high or very low enzyme activities. To keep the viscosity change within convenient limits, the amount of enzyme must be increased or decreased. In order to obtain comparable results in such cases, it was necessary to establish the relation between the rate of gelatin liquefaction and the volume of enzyme solution used. The results are given in table 1.

EXPERIMENTAL

The inhibition of the proteolytic enzymes of a number of anaerobic spore-formers by fresh, normal rabbit serum was investigated. The results are given in table 2.

No non-pathogenic organism in the above list possesses proteolytic enzymes which are able to resist inhibition by normal serum. Of the pathogens tested, *Clostridium histolyticum*, *C. welchii*, and *C. oedematis-maligni* possess proteolytic enzymes which are capable of hydrolyzing proteins in the presence of normal serum. It is probable that this characteristic is an important one in pathogenesis, enabling the members of this group to establish a foothold in the body of the host and to obtain those breakdown products of protein which are their main source of energy. *C. botulinum*, although usually considered as pathogenic, nevertheless is so only by virtue of its toxin, which must be produced *ex vivo*.

TABLE 2

Inhibition of the proteinases of Clostridia by normal rabbit serum

ORGANISM	ENZYME ACTIVITY	ACTIVITY WITH 0.1 ML. SERUM
C. aerofetidum	23.5	<1
C. botulinum	7.5	<1
C. fallax	1.9	<1
C. histolyticum R-	35.0	3.0
C. histolyticum S-	33.0	33.0
C. oedematis-maligni	2.0	2.0
C. putrificum	22.0	3.0
C. sporogenes	45.0	<1
C. welchii	15.0	12.0

Pozerski and Guelin (1938) reported that the escharotic properties of culture filtrates of the spore-forming anaerobic bacteria bore little or no relation to their proteolytic activity. They did not, however, determine whether there was any inhibition of the proteolytic enzymes by normal serum, although they did report, in a separate paper, that the proteinases of *Clostridium sporogenes*, *C. bifermentans*, and *C. sordelli* were inhibited by egg albumin, while those of *C. histolyticum* were not.

Heiden (1905, 1906) reported that trypsin was inhibited by serum albumin. This inhibition was studied in detail by Hussey and Northrup (1923), who concluded that the inhibition was due to the relative indigestibility of the serum albumin, to which the trypsin became attached. It was thought that the "anti-trypsin"

of the serum might be the substance responsible for the inhibition of certain of the bacterial proteinases. In making the comparison of the inhibition of trypsin and a bacterial proteinase which was inhibited by normal serum, the filtrate of a rough strain of *C. histolyticum* was used. The proteinase of this organism was active in liquefying gelatin, was almost totally inhibited by fresh serum, and was readily obtainable.

Serum which had stood for about a week at room temperature, or for two or three months in the refrigerator, inhibited the bacterial proteinase hardly at all, while it did inhibit trypsin almost as well as fresh serum. Half saturation with ammonium sulfate also served to destroy the ability to inhibit the bacterial proteinase, but did not affect the "anti-tryptic" activity.

These results made it seem possible that there might be two substances in serum, one inhibiting trypsin and the other inhibiting the bacterial proteinase. However, all attempts to separate the two were unsuccessful. Both were destroyed by heat at the same rate, being half destroyed by an exposure of 10 minutes at 64°C., and being almost completely destroyed by an exposure of 30 minutes at this temperature.

Serum was separated into its constituent fractions by electrophoresis in the Tiselius apparatus.[1] These fractions were tested for the inhibition of trypsin, and for the inhibition of the proteinase of the rough strain of *C. histolyticum*. The results are given in table 3.

The inhibition of the bacterial proteinase by the β globulin could not be represented quantitatively. There was an almost complete inhibition of gelatin liquefaction for thirty minutes. At the end of this time, liquefaction began and proceeded almost as rapidly as if no inhibiting substance were present. The activation shown by the Δ globulin, although slight, was consistent. It was also found with globulin prepared from normal horse serum.

The parallelism of the inhibition of trypsin and of the bacterial proteinase by different fractions of normal rabbit serum, as shown

[1] The authors are indebted to Dr. Laura Krejei and Mr. Robert Jennings for preparing the serum fractions.

in table 3, indicates that the inhibition of these two enzymes is primarily due to a single substance, the albumin of the serum, although the β globulin inhibited the bacterial proteinase more than it did the trypsin. Serum albumin prepared in the Tiselius apparatus is submitted to a minimum of manipulation and exposure to adverse conditions, and contains no more than traces of the other protein constituents. However, if the albumin of the serum is responsible for the inhibition of both of these enzymes, then certainly the mechanism of inhibition cannot be the same. It is possible that certain labile groups which can be altered by treatment with ammonium sulfate or by standing at room temperature, are responsible for the inhibition of the bacterial proteinase, but have no effect on the inhibition of trypsin.

Immune serum prepared by the injection of filtrates of *C. histolyticum*, smooth, into rabbits, inhibited the enzymes of this

TABLE 3

Inhibition of bacterial proteinase and trypsin by serum fractions

ENZYME	ORIGINAL ACTIVITY	WHOLE SERUM	ALBUMIN	β	$\beta + \gamma$	$\gamma + \Delta$	Δ
Trypsin..................	17	<1	<1	13	10	15	18
C. histolyticum R-........	13	<1	<1	1–14	7	13	15

organism. Inhibition of the proteolytic enzymes of bacteria by immune sera has been reported by Bertiau (1914) for *Pseudomonas aeruginosa*, Blanc and Pozerski (1920) for *C. histolyticum*, Dukes (1922) for *P. aeruginosa*, and Grassmann (1938) for *C. welchii*.

The inhibition of the proteinases of *C. histolyticum* was specific, the serum of the rabbit immunized to *C. histolyticum* inhibiting the proteinase of this organism, but not that of *C. sporogenes*, *C. welchii*, or even that of rough strains of *C. histolyticum*. This experiment was performed with antiserum which had stood sufficiently long in the cold to have lost its ability to inhibit the proteinases of the non-pathogenic organisms.

The inhibiting factor in this serum was probably antibody, since it was associated with the globulins of the serum, being precipitated by half saturation with ammonium sulfate. It was

fairly stable, being present in practically full amount in serum which had been kept sterilely in the cold for over a year.

Dukes considered that the inhibition of the enzymatic activity was not due to an antibody which inactivated the enzyme, but rather to a precipitin which removed the enzyme from solution, and consequently, from contact with the substrate. In our experiments with immune sera, no visible precipitate was ever formed. While this does not invalidate Dukes' contention, nevertheless, the sharp specificity shown by the anti-enzyme sera indicates strongly that these enzymes are capable of acting as specific antigens, and of inducing the production of specific antibodies.

SUMMARY

1. The proteolytic activity of culture filtrates of various spore-forming anaerobes was determined by the gelatinase method of Haines, so modified as to permit the determination of gelatinase in solutions of widely differing enzymatic activity.

2. Fresh, normal rabbit serum inhibited the proteinases of *Clostridium aerofetidum*, *Clostridium botulinum*, *Clostridium fallax*, *Clostridium putrificum*, *Clostridium sporogenes*, and a rough strain of *Clostridium histolyticum*. The β globulin and albumin of the serum were responsible for this inhibition, the latter accounting for the most of it. Trypsin was inhibited only by the albumin.

3. Normal serum did not inhibit the proteinases of *Clostridium histolyticum*, *Clostridium oedematis-maligni*, or *Clostridium welchii*.

4. Immune serum specifically inhibited the proteinase of *C. histolyticum*.

The authors wish to express their appreciation to Dr. Ellice McDonald, Director, for his interest and encouragement in this work.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MISSOURI VALLEY BRANCH

UNIVERSITY OF KANSAS, LAWRENCE, DECEMBER 3, 1938

BIOLOGICAL AND CHEMICAL STUDIES OF THE SEROLOGICAL TESTS USED IN THE DIAGNOSIS OF SYPHILIS. *Noble P. Sherwood, Glenn C. Bond, and Harold F. Clark*, Department of Bacteriology, University of Kansas, Lawrence.

This investigation represents a number of objectives as follows: (1) A study of the distribution of "reagin-like" substances in the blood of beef, sheep, horse, rabbit, guinea pig, dog, hog, chicken, and snakes by means of routine Kolmer-Wassermann, Kahn, Kline, and a "wide-spread modification of the Kahn" techniques. In the latter test standard Kahn antigen was used and ratios of serum to antigen varying from 100:1 to 0.19:1 were employed. The ratios used in the 3-tube Kahns were included in the series. Positive serological findings were obtained quite frequently with all species studied except the guinea pig. The Kolmer was less frequently positive than the flocculation tests. (2) Using Howe's method of fractioning beef and horse serum it was ascertained that the "reagin-like" substance was present in either or both the euglobulin and pseudoglobulin I fractions and it was destroyed at about the same temperature as syphilitic reagin. (3) When various animal sera and sera from untreated and treated cases of syphilis were studied by means of the special flocculation technique to determine optimum ratios or zones of flocculation, certain rather striking results were obtained:

(1) In untreated cases of secondary syphilis where the reagin titre is high the *optimum zone* of flocculation was not infrequently between 20:1 and 0.25:1. In one case of untreated tertiary syphilis it was between 6:1 and 0.75:1. In treated cases there was a definite shift toward the higher ratios and a narrowing of the zone. A few clinically cured ones were negative in all ratios while others that were negative in the diagnostic ratios gave strong flocculation in higher ratios, i.e., 12:1.

(2) The zones of flocculation observed with animal sera resembled the ratios in many treated cases. This indicates a low titre of the "reagin-like" substance.

RESISTANCE OF RABBITS TO LARVAL CESTODES. *A. B. Leonard*, Dept. of Zoology, University of Kansas.

Normal young rabbits were fed approximately 2000 eggs of *Taenia pisiformis*, and the tissue responses in the liver studied. A typical inflammatory reaction occurs, resulting in the walling off of many of the larvae so completely that further development is impossible.

An equal group of rabbits was fed a similar dosage of eggs and at the same time given intravenously 55 ml. of

serum taken from rabbits carrying a heavy infection of larval cestodes. The tissue response was studied as before.

Results may be summarized as follows:

1. In normal young rabbits, approximately 500 larvae began development in the liver, the tissue reaction accounting for the death or definite inhibition of growth in all but about a tenth of the total number. An average of 55 larvae per rabbit continued normal development.

2. In rabbits receiving the serum from infected rabbits, at the time of infection, an average of 50 larvae began development in the liver. Only an occasional larva was able to continue normal development.

3. The tissue response in the normal rabbit results in walling off the larvae at the end of a period of 16 to 21 days. In immunized animals, the tissue response was greatly accelerated, and complete enclosure of the larvae was accomplished in 6 to 7 days.

THE INFLUENCE OF CERTAIN CHEMICAL SUBSTANCES UPON THE GROWTH OF LEGUME BACTERIA. *T. M. McCalla*, Dept. of Bacteriology, Kansas State College, Manhattan, Kansas.

Elucidation of the phenomena of growth stimulation of legume bacteria by various plant extracts is needed. Attempts at partial purification of plant extracts have resulted in a limited chemical characterization of the growth-stimulating substance. It is now known that definite chemical substances promote growth of certain bacteria. This study attempts to determine the value of some of these substances upon the growth of legume bacteria.

Additions of chlorophyll, indol butyric acid, phenyl lactic acid, indol lactic acid, and nicotinic acid in concentrations of 1:1000 and 1:10,000 to calcium gluconate medium did not seem especially beneficial to normal alfalfa organisms as judged by the amount of growth produced on slants. Pimelic acid and B-alanine in concentration of 1:100, 1:1000, and 1:10,000 stimulated growth of alfalfa organisms almost equal to that obtained by addition of various plant extracts.

HETEROAUXIN PRODUCTION BY ROOT NODULE BACTERIA. *Carl E. Georgi*, Department of Bacteriology, University of Nebraska, Lincoln, Nebraska.

Differences in rate and amount of beta indol acetic acid produced by efficient and inefficient strains of rhizobia were determined, in an effort to demonstrate the significance which heteroauxin may play in the formation of nodules in the roots of legumes. Inefficient strains, in some cross inoculation groups, were found to produce slightly more auxin than did corresponding efficient strains. A microorganism commonly found in root nodules, *Achromobacter radiobacter*, produced heteroauxin in concentrations greater than did the rhizobia, yet it is unable to incite nodule formation on legumes.

TEMPERATURE RELATIONSHIPS OF SOME COMMON INTESTINAL NON-SPORULATING ANAEROBIC BACTERIA. *Keith H. Lewis*, Department of Bacteriology, University of Nebraska, Lincoln, Nebraska.

Studies of the growth temperature relationships of 162 strains of non-sporulating, anaerobic, rod-shaped bacteria isolated from the intestinal tract show that the optimum for the entire group lies between 35° and 40°; the maximum falls slightly above or

below 45°; and the minimum growth temperature varies from 10° to 30°C. depending upon the type of strains used.

The time required to kill these organisms by exposure to 60°C. varies from less than five minutes to more than half an hour. In general, the gram-positive strains, except *Bacteriodes bifidus*, are more resistant than the gram-negative types.

Correlation of minimum growth temperature and thermal resistance with morphological, cultural and biochemical characteristics suggests the desirability of utilizing these factors in the classification of this group.

Investigations of the Use of the Resazurin Test for Grading Milk. *F. E. Nelson, Kansas State College.*

Of the four different brands of resazurin compared, only two have been found to be suitable for use in the resazurin test. The other two brands were abnormal with respect both to the initial color imparted to the milk and the sequence of color changes during incubation. Standardization and probably certification of the dye is indicated.

Using aseptically drawn milk samples from individual quarters of cows in the Experiment Station herd, some correlation was found between the numbers of leucocytes present and the sequence of color changes of the dye. No correlation was found between the observed color changes of the dye and the presence of mastitis streptococci, as determined by either the Hotis test or the direct microscopic examination of incubated aseptically drawn samples.

When the resazurin test was used in the grading of market milk samples, the results indicated that a variety of factors were operative in determining the course of the reduction of the dye. Three different ways of reading the test were used, the first being the color change at one hour, the second being the color change at three hours and the third being the time required for the dye to change to the pure pink stage in its reduction. The standard plate counts, the direct microscopic counts and the leucocyte counts are used as criteria for evaluating the test. More standardization of the test is required.

A Report on the Hotis Test. *V. D. Foltz,* Department of Bacteriology, Kansas State College.

In the routine control of mastitis in the dairy herd at the college, periodic examination within at least monthly intervals is made of each quarter of all cows. These quarter samples are collected under practically aseptic conditions. On the basis of microscopic examination of incubated samples of this milk, the cows are grouped into three classes as follows:

A Class: Supposedly free from mastitis, leucocyte count consistently below 500,000 per ml.; no long-chained streptococci.

B Class: Suspicious or doubtful for mastitis; leucocyte count of one or more quarters consistently above 500-000 per ml.

C Class: Supposedly positive for mastitis; leucocyte counts of the milk from one or more quarters usually though not necessarily above 500,000 per ml.; long-chained streptococci observed upon microscopic examination of the milk from one or more quarters of the udder after incubation of the sample for 16 hours at 37°C.

Using the above procedure, the Hotis test has been run on duplicate quarter samples of milk. In order to evaluate this test the microscopic examination of incubated samples has been used as a basis of comparison. Some 7640

quarter samples have been studied of which 4672 were "A" cow quarter samples, 1801 were "B" cow quarter samples, and 1167 were "C" cow quarter samples.

In the "A" cows 4 false positive Hotis tests were recorded in comparison to 25 false positives in "B" class cows. In the "C" class group 67 false positive Hotis tests and 87 false negative Hotis tests were observed.

A study of these results shows that the Hotis test was in agreement with the microscopic examination in 97.6% of all tests. However, in a study of the results of the "C" class cows alone, which furnished 1167 of the 7640 quarters examined, a positive Hotis test was given in but 84.3% of those samples exhibiting long-chained streptococci on incubation. On the other hand, only 89.1% of the samples which were negative on the incubated samples were likewise negative for the Hotis test.

In spite of these errors the Hotis test appears to be the most satisfactory of any test developed to date for the field diagnosis of streptococcus mastitis.

Some Studies on Slow-lactose-fermenting Bacteria. *L. D. Bushnell*, Department of Bacteriology, Kansas State College.

During the study of certain paratyphoid infections in chickens we isolated numerous coliform organisms giving acid and gas in glucose but no change in lactose within 48 hours and not agglutinated by any of the antiparatyphoid sera. Such organisms are neither typical colon nor typical paratyphoids. They do not appear to be coli-aerogenes intermediates and should probably be classed as paracolon bacilli.

These organisms were found to include certain mutable types, which form secondary colonies, and others which appear to be similar but which are non-mutable. Both exhibit delayed lactose fermentation. This characteristic delays their identification. Such organisms may be differentiated from the Salmonellas by use of tests for indol, H_2S and ability to grow rapidly on Simmon's citrate agar. The paracolon organisms which we have studied all produce indol, but none grow on citrate agar or produce H_2S. None of the paratyphoids which have been isolated during this investigation produced indol but all grew rapidly on citrate agar and produced large amounts of H_2S.

It was not possible to correlate the presence of the paracolon group in the intestine of the chicken with any particular type of disease but they seem to appear most frequently in certain flocks. Our investigations have not made it possible to explain the reason for their appearance under these conditions.

CENTRAL PENNSYLVANIA BRANCH

Annual Spring Meeting, The Gilliland Laboratories, Marietta, Pa., May 13, 1939

Plant Nutrition and Disease Resistance. *J. Naghski, R. G. Harris, D. E. Haley and J. J. Reid*, The Pennsylvania Agricultural Experiment Station.

A study has been made of the relation between variations in the nitrogen /mineral uptake of plants and variations in resistance to disease. It has been a common assumption that, other

things being equal, the higher the nitrogen level of the plant, the greater the susceptibility of the plant to disease. Data obtained by the authors indicates that this is not the true relation between nitrogen uptake and disease susceptibility.

The results indicate that it is not a question of how much nitrogen a plant absorbs within reasonable limits, that determines susceptibility, but the stage in the life of the plant when significant quantities of nitrogen are absorbed.

Tobacco plants grown with adequate mineral fertilization may mature with a nitrogen content in excess of four per cent and show no significant loss in resistance to leafspot diseases if the nitrogen is supplied without material fluctuations during the stage of active growth. On the other hand it has been found that tobacco plants may show extreme susceptibility to leafspot diseases with a nitrogen content of less than two per cent, if the nitrogen is supplied erratically with significant amounts available during the ripening stage.

It is considered inadvisable, therefore, to employ fresh animal manure, old legume residues and other sources of nitrogen which may provide a fluctuating and late supply of this element in the fertilization of tobacco and other short season crops.

DISSIMILATION OF GLUCOSE BY MEMBERS OF THE GENUS BACILLUS. *F. H. Gallagher and R. W. Stone,* The Pennsylvania State College.

Studies were made of the dissimilation of glucose by *Bacillus subtilis, Bacillus mesentericus,* and *Bacillus vulgatus.* All species showed phosphorylation in the early stages of fermentation. Analysis of the fermentation showed the presence of acetic acid, butyric acid, ethyl alcohol, acetyl methyl carbinol, and carbon-dioxide, in all cases. These compounds were determined quantitatively.

With M/50 sodium fluoride *Bacillus subtilis* showed a higher yield of acetic acid than that obtained in the control. With M/25 sodium fluoride there was a further increase in the amount of acetic acid formed, and a decrease in acetylmethylcarbinol production. *Bacillus vulgatus,* in the presence of M/100 and M/50 sodium fluoride, and *Bacillus mesentericus,* in the presence of M/50 sodium fluoride, gave similar results.

All three species gave comparative results as to phosphorylation, types of end products, and amounts of these products.

THE PHOTOLOMETER AND ITS USE FOR THE QUANTITATIVE DETERMINATION OF NITROGEN. *Paul E. Portner,* Gilliland Laboratories, Inc.

MICROORGANISMS ATTACKING MEDIUM WEIGHT LUBRICATING OILS. *A. G. C. White and R. W. Stone,* The Pennsylvania State College.

THE RELATION OF THE SOLUBLE SPECIFIC SUBSTANCE TO VIRULENCE AND SPECIFICITY IN BACTERIAL LEAFSPOT ORGANISMS. *R. G. Harris, J. Naghski, M. A. Farrell and J. J. Reid,* The Pennsylvania Agricultural Experiment Station.

Laboratory animals have been immunized by the authors with cultures of *Pseudomonas fluorescens* and the cultures then cultivated in the presence of the homologous antiserum until "R" forms were secured. Animals were then immunized with the "R" forms and the "R" cultures were cultivated in the presence of the homologous antiserum and killed cells of *Phytomonas tabaci.* "S" forms were obtained in this manner which proved to be cul-

turally and serologically identical with strains of *Phyt. tabaci*. Studies of infective ability showed these "S" forms to be identical with *Phyt. tabaci* in the nature of the lesion produced on the tobacco plant.

In a similar manner "R" forms of *Phyt. tabaci* were secured and cultivated in the presence of the homologous antiserum and killed cells of *Ps. fluorescens*. The "S" forms produced in this manner were culturally and serologically identical with the strains of *Ps. fluorescens* which had been killed and used in the culture medium. These "S" forms were unable to produce wildfire as proved by infective studies.

Agglutinin absorption studies showed the "R" strains produced from *Ps. fluorescens* and *Phyt. tabaci* to be serologically identical.

Work with several other members of the genus *Phytomonas*, including *Phyt. angulata*, *Phyt. cerasi*, *Phyt. primulae*, and *Phyt. vignae*, showed the same relationship to exist between these organisms and *Ps. fluorescens*.

It is concluded that specificity and virulence are associated with the nature of the soluble specific substance in this group of organisms.

SOME EFFECTS OF SULFAPYRIDINE ON PNEUMOCOCCUS TYPE I. *Roger D. Reid*, Laboratories of Bacteriology, Johns Hopkins Medical School, Baltimore.

In vitro and *in vivo* experiments with the pneumococcus Type I in the presence of sulfapyridine indicated that the drug had no appreciable effect on the phagocytosis of pneumococci. There were no changes in morphology or somatic elements of the cell observed. No dissociation or changes in colony appearance were detected after growing the organisms in the presence of 10 mgm. % of the drug or when the drug was added to the medium used for plating the organisms. The drug did not affect the capsule either *in vivo* or *in vitro* and the "quellung" reaction was not interfered with.

A bacteriostatic and slight bactericidal effect was noted and it is presumed that the bacteriostatic action is responsible for the beneficial effects of the drug in pneumococcus infection.

OHIO BRANCH

OHIO STATE UNIVERSITY, COLUMBUS, MAY 27, 1939

SULFAPYRIDINE, SULFANILAMIDE AND ANTISERUM IN THE TREATMENT OF EXPERIMENTAL PNEUMOCOCCUS PNEUMONIA. *L. H. Schmidt and Carolyn Hilles*, The Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati.

Rats were infected with types I or II *Diplococcus pneumoniae* by intrabronchial inoculation. At varying intervals after infection groups of these animals were treated with sulfapyridine, sulfanilamide or antipneumococcus serum. These treatments were repeated at daily intervals for 7 days; surviving animals were sacrificed 14 days after infection.

In both types I and II infections sulfapyridine, sulfanilamide and antiserum had marked curative action when administered 6 and 12 hours after infection. In type I infections the three therapeutic agents seemed to be equally effective; in type II infections

sulfanilamide and sulfapyridine were slightly more effective than antiserum.

Sulfanilamide, sulfapyridine and antiserum had considerable curative action when administered as long as 24 hours after infection, although their effectiveness was considerably less at this time than at 12 hours. In type I infections the curative action of antiserum was greater than that of either sulfapyridine or sulfanilamide, which were equal in effectiveness. The results in type II infections were in contrast to this, for sulfanilamide had a much greater curative action than either antiserum or sulfapyridine.

Complications Following Sulfapyridine Therapy in Experimental Animals. *John A. Toomey, Herbert S. Reichle and William S. Takacs*, Division of Contagious Diseases, City Hospital, Cleveland and the Department of Pediatrics, Western Reserve University, Cleveland.

Complications of the urinary tract resulted from sulfapyridine therapy given Rhesus monkeys which had been injected with poliomyelitis virus and treated with specific horse antiserum. These complications were hydronephrosis and dilatation of the urinary bladder due to the formation of sulfapyridine crystals arranged as calculi in the ureters and urethra.

Some of the animals thus treated with sulfapyridine and specific serum developed quadriplegia sooner than did the controls. Since retention is often observed in animals infected with poliomyelitis, the obstruction due to sulfapyridine may increase the absorption of poliomyelitis virus.

Administration of Rabbit Antipneumococcal Serum in Relation to Human Blood Groups. *E. E. Ecker*, Institute of Pathology, Western Reserve University, Cleveland.

Effects of Lithium Chloride on the Variation, Growth, and Oxygen Consumption of Escherichia communior. *Louis V. Blubaugh*, The William S. Merrell Company, Cincinnati.

The Effects of X-Rays on the Dissociative Rates of Certain Bacteria. *Sol. Haberman and Louis D. Ellsworth*, Departments of Bacteriology and Physics, Ohio State University, Columbus.

Broth cultures and water suspensions of *Staphylococcus aureus* and *Serratia marcescens* were exposed to the emanations from copper and molybdenum x-ray tubes. The cells were then transferred to fresh nutrient broth and incubated. From time to time the cultures were streaked on nutrient agar plates and the types and numbers of variants were observed.

The treated cells had a tendency to produce a larger number and variety of variant forms than untreated controls. Furthermore, some of the dissociants isolated from treated cells showed a marked ability to produce further dissociations in relatively short periods of time. Some of the colonial forms resulting from irradiated cultures were found also in the controls. Cultures of *Staphylococcus aureus* that had never been exposed to gelatin, mannite, lactose, raffinose and salicin, and could not attack them with detectable changes, produced variants that were able to utilize these substances as foods.

On rare occasions, irradiated staphylococcus cells resulted in streptococcus-like forms which appeared in the cultures within 18 hours after x-ray treatment. The chains were composed of oval pairs of cells, often numbering over 100 to each chain.

Exposure to x-rays speeds up the rate of dissociation and hence is a

valuable tool in the study of bacterial inheritance.

Lung Changes in the Guinea Pig Fetus Infected with the Human Influenza Virus. *N. Paul Hudson and F. W. Gallagher*, Department of Bacteriology, Ohio State University, Columbus.

An Attempt to Identify Cultures of Shigella paradysenteriae Using Specially Prepared Sera. *Barbara Johnson and Merlin L. Cooper*, Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati.

Rabbit agglutinating sera were prepared against 3 strains of *Shigella paradysenteriae* isolated from the stools of infants and children in Cincinnati during the summer of 1937. Agglutinating antigens were also prepared choosing 7 strains of *Shigella paradysenteriae* isolated in the same summer. In the study of the diarrhea patients in the summer of 1938 the stools of 101 out of 207 patients were found to contain organisms belonging culturally to the *Shigella paradysenteriae* group. In 63 cases the patients' organisms were agglutinated by one of the prepared antisera. In 37 cases the patients' sera agglutinated one or more of the prepared antigens to a titer of at least 1:80 and in 41 cases the patients' sera agglutinated their own organisms to at least that titer. The patients' sera contained the largest amount of agglutinins for both their own and the prepared antigens during the second, third, and fourth weeks after onset of the diarrhea.

It was found that the serological type of the patients' sera and the *Shigella paradysenteriae* isolated from the same patient were often the same.

An Accidental Laboratory Infection with Shigella Dysenteriae (Shiga). *Oram C. Woolpert and Homer F. Marsh,* Departments of Bacteriology and Medicine, Ohio State University, Columbus.

A case is presented concerning an infection in a student resulting from a laboratory accident during the injection of a rabbit with a living culture of *Shigella dysenteriae (Shiga)*.

The organism had been isolated from a case of dysentery only three months previously.

The precise time of exposure was known; the onset of symptoms established the incubation time at almost exactly 48 hours.

Cultural examination of the stools on the first, second, and seventh days of hospitalization yielded almost pure growths of *Shigella dysenteriae*.

The organism was also identified serologically within 24 hours of the patient's admission by utilizing the culture obtained directly from the first plates and antiserum prepared by the student for the very strain to which he was exposed.

Serologic studies of the patient's serum showed that until the thirty-eighth day after exposure, no agglutinins were present for *Shigella dysenteriae*. The titer at 38 days was 1:80. No agglutinins were demonstrated for *Shigella paradysenteriae (Flexner)* in control tests.

Apparently, this is the first recorded case of dysentery due to *Shigella dysenteriae* resulting from a laboratory infection.

Correlation of the Schick Test and the Blood Serum Antitoxin (Diphtheria) in Scarlet Fever Patients. *Carl E. Duffy*, Children's Hospital Research Foundation and

the Department of Pediatrics, University of Cincinnati, Cincinnati.

Twenty-two scarlet fever patients, ranging in age from 2 to 13 years, were studied in an attempt to correlate the diphtheria antitoxin content of their sera with the Schick test. Eight or 36 per cent were Schick positive and none of these had as much as 0.002 unit of antitoxin per milliliter of serum. Fourteen or 64 per cent were Schick negative and all except 1 had more than 0.002 unit of antitoxin per milliliter of serum.

An attempt to evaluate the antitoxin directly by injecting intradermally a constant increment (1:50 M.L.D.) of toxin at three day intervals gave negative results except in three patients. These patients had low serum antitoxin values and gave reactions to more toxin than that contained in the ordinary Schick test dose.

THE EFFECT OF THE ADDITION OF HETEROPHILE ANTIGEN ON THE IMMUNOLOGIC PROPERTIES OF SERA OF RABBITS INJECTED WITH DIPLOCOCCUS PNEUMONIAE. *Sol Miller and D. Frank Holtman*, Department of Bacteriology, Ohio State University, Columbus.

Bailey and Shorb reported the results of mouse protection tests which indicated that the protective properties of antipneumococcic sera may be increased from 4 to 8 times when the specific sera are combined with heterophile serum. Although mice are members of the "guinea pig" group, Hyde showed that they are relatively non-susceptible to large amounts of heterophile antibody.

Three groups of rabbits were immunized during the present investigation. One group was injected with pneumococcus type III, another with heterophile antigen in the form of horse serum, and a third with pneumococcus type III and horse serum.

Antisera thus induced were tested for demonstrable antibodies and for protection of mice infected with pneumococcus types I, II or III.

Sera containing pneumococcus type III antibody alone, or a mixture of pneumococcus type III and heterophile antibodies, protected mice against the specific organism, but failed to protect against types I and II infections. Heterophile antibody, as induced by rabbit injection of horse serum, gave no protection to mice against infection with any of the three types. The addition of sera, containing heterophile antibody alone, to sera containing pneumococcus type III antibody did not increase the protective action of the antipneumococcic sera for mice.

EASTERN NEW YORK BRANCH

DIVISION OF LABORATORIES AND RESEARCH, ALBANY, MAY 26, 1939

STUDIES ON MUCOID VARIANTS OF ESCHERICHIA COLI. *Carl A. Lawrence*, Research Laboratories, Winthrop Chemical Company, Inc. Rensselaer, N. Y.

A strain of *Eschcrichia coli* is described which develops on agar plates as true rough colony forms when incubated in the hot room (37°C.), which, upon subsequent storage of the plates at room temperature, gives rise to mucoid outcroppings and borders surrounding the periphery of the parent R colonies. Transplants of material from the mucoid areas to freshly poured agar plates with incubation at

the lower temperature, resulted in the development of pure mucoid colonies. Similar plates placed at 37°C. give rise to colonies which are not grossly unlike the parent (R) forms.

The mucoid variant develops on certain differential media as a profuse, striated, moist mucoid growth which proliferates to the extent where it will eventually cover the entire plate. The media giving rise to this profuse type of growth contain but 1 per cent agar.

The presence of a carbohydrate, low concentration of agar in the media, and a relatively low temperature (23°–26°C.) are necessary for the growth of the mucoid colony type in the profuse striated form.

The purified carbohydrate material isolated from the profuse mucoid type of growth does not exhibit the properties usually associated with a haptene. The substance has been obtained entirely free of any demonstrable traces of nitrogen.

THE QUANTITATIVE DETERMINATION OF THE OPTIMAL RATIO OF CHOLESTEROL TO TISSUE EXTRACT IN THE COMPLEMENT-FIXATION TEST FOR SYPHILIS. *John Kent*, Division of Laboratories and Research, New York State Department of Health, Albany.

A linear relation between syphilitic serum and complement is observed when antigen is present in optimal concentrations. This relation has been used as a quantitative measure of changes in antigen activity due to variation in the ratio, cholesterol to extract. To portions of extract undiluted and diluted in 1, 2, and 3 parts of alcohol, cholesterol was added in amounts between 0.1 and 1.0 per cent. Anticomplementary and nonspecific properties were minimal.

With increase to an optimum in the ratio, cholesterol to extract, increase to maximum antigenic activity was observed. Further increase resulted in identical or diminished activity.

Maximum activity exhibited by cholesterol and extract in optimal ratio was identical whether extract was diluted in 1, 2, or 3 parts of alcohol before cholesterolization. With undiluted extract, increase in the ratio within the range of cholesterol solubility resulted in increased activity; since cholesterol in larger amounts was insoluble, an optimal ratio could not be determined. Failure of previous investigators to demonstrate an optimal ratio may be traced to (1) unsatisfactory extracts which, with cholesterol in high concentrations, exhibited anticomplementary or nonspecific activity, or (2) failure to use extracts in concentrations in which an optimal ratio, cholesterol to extract, might be demonstrated.

CHANGES IN THE OXIDATION-REDUCTION POTENTIALS OF THE SKIN OF GUINEA PIGS ON A SCORBUTIGENIC DIET. *Calvin C. Torrance*, Division of Laboratories and Research, New York State Department of Health, Albany.

It has been shown that following the injection of lethal doses of diphtheria toxin the ascorbic-acid concentration of the suprarenal glands of guinea pigs is markedly diminished. Similarly, the vitamin-C concentration of the skin is reduced when inflammatory lesions are induced in it by either diphtheria toxin or heat. Changes in the oxidation-reduction potentials of the skin occur under both conditions. In order to obtain data on the interrelationship of these changes, guinea pigs were placed on a scorbutigenic diet. The

ascorbic-acid concentration and Eh of the skin and the amount of ascorbic acid in the suprarenals were determined by sacrificing animals at intervals for a period of twenty-seven days. While there appeared to be a close parallelism between the vitamin-C concentration of the suprarenals and of the skin, no constant relationship between the amount of the vitamin C present and the oxidation-reduction potentials of the skin was demonstrated.

Survival of Gonococci in Collodion Sacs in Rabbits. *A. H. Harris*, Division of Laboratories and Research, New York State Department of Health, Albany.

In this study the stock strain of gonococcus used was isolated from a case of ophthalmia neonatorum. It will grow at 39°C. The growth from a 48-hour slant, suspended in 50 ml. of human ascitic fluid, filled 12 sacs of 10.3-per-cent collodion (J. Bact., in press; J. Bact., 1939, 37, 229). Six were introduced into the peritoneal cavity of a rabbit. Six were dropped into tubes of beef-infusion broth. Of the latter, three were incubated at 34°C. and three at 39°C.; in some, the microorganisms grew through the sac walls and multiplied in the surrounding broth.

The rabbit remained afebrile. Weekly quantitative complement-fixation tests using gonococcus antigen revealed only a very slight rise in serum titer by the end of thirty-eight days when the experiment was ended. The *in vivo* sacs were found wrapped in folds of omentum and covered with fibrin which when cultured yielded no growth. Microscopic examination of slide preparation showed blood cells, mostly macrophages, enmeshed in the strands. The sacs contained turbid fluid; films revealed debris and less than one typical diplococcus per oil-immersion field, but no fibrin or blood cells, even in the centrifugalized sediments. The latter were introduced into the conjunctival sacs of a rabbit; no detectable reaction occurred. The contents of the 6 *in vivo* sacs yielded on subculture a luxuriant pure culture of gonococci, confirmed by carbohydrate fermentation. The results are in accord with those of deChristmas (Ann. inst. Pasteur, 1897, **11**, 609).

The Activity Against Type-VIII Pneumococcus of an Enzyme Produced by a Soil Microorganism Grown on Type-III Polysaccharide. *Grace M. Sickles and Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

A soil microorganism has been isolated which closely resembles in morphology and cultural characters our previously described strains of *Rhodobacillus palustris*. The earlier strains were highly specific in their activities, one utilizing type-III, the other type-VIII pneumococcal carbohydrate only, but the new strain decomposes both types III and VIII polysaccharides.

In mineral medium containing type-III pneumococcal polysaccharide, it produces enzymes active against pneumococcal carbohydrates types III and VIII. Much of the antitype-III activity is lost in Berkefeld filtration; the antitype-VIII enzyme is freely filterable. Such enzyme preparations concentrated on a 6½-per-cent nitrocellulose membrane proved to have a high protective value against type-VIII pneumococcal infections in mice.

If grown on type-VIII carbohydrate, it produces only an antitype-VIII enzyme.

When a filtered antitype-III enzyme preparation, previously prepared by the activity of one of the original specific antitype-III *Rhodobacillus palustris* strains on type-III carbohydrate, was inoculated with one of the earlier specific antitype-VIII *Rhodobacillus palustris* strains, an antitype-VIII enzyme was produced, while the original antitype-III enzyme was retained.

Apparently, the splitting of the type-III polysaccharide produces a fraction which, although it does not react serologically with type-VIII serum, serves as a suitable substrate for the production of antitype-VIII enzyme.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND THIRTY-FIFTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, JANUARY 24, 1939, PHILADELPHIA, PA.

A NEW FILTERABLE AGENT ASSOCIATED WITH RESPIRATORY INFECTIONS.

Joseph Stokes, Jr., *Hobart A. Reimann* and *Dorothy R. Shaw*, Department of Pediatrics, School of Medicine, University of Pennsylvania and The Children's Hospital of Philadelphia, Philadelphia, Penn.

A filterable agent was isolated from throat secretions of a patient with an upper respiratory infection (Reimann, 1938).[1] Francis and Magill (1938)[2] isolated independently from the same patient a comparable agent.

These secretions were inoculated intranasally into a ferret, which became comatose in 24 hours. Brain and lung emulsions were filtered separately through Berkefeld V filters. This agent inoculated intranasally and intracerebrally into mice and guinea-pigs caused in 10–20 days weakness, loss of appetite and weight, and in almost all instances paresis of the hind legs. The lungs showed grossly a bluish-gray, firm, patchy bronchopneumonia.

Brain showed toxic reaction and degeneration of the cells in the deeper cortical layers and of the ganglion cells in the medulla. In the lungs there was found an inflammatory exudate, having a lobar and peribronchial distribution. This consisted mainly of mononuclear cells and fibrin with clumps of neutrophils. The bronchiolar epithelium was swollen and redundant. The fibromuscular wall of the bronchi and bronchioles was thickened and highly vascular (Wolman, 1938).[3]

Cultures of filtrates of brain and of lung emulsions showed no evidence of bacterial contamination. The agent passed through a collodion membrane of 780 $m\mu$. average pore diameter (Francis and Magill, 1938).[2]

Whether this agent was of animal or human origin, it is impossible to state. However, the findings suggest that a new filterable agent has been described which causes disease in mice, guinea-pigs and possibly ferrets, and it is also possible that it may have been in part responsible for the disease of the patient.

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[1] Reimann, Hobart A., 1938, An acute infection of the respiratory tract with atypical pneumonia. J. Am. Med. Assoc., 111, 2377–2384.

[2] Francis, T., jr., and Magill, 1938, personal communication.

[3] Wolman, I. J., jr., 1938, personal communication.

FURTHER STUDIES ON THE VALUE OF ROUTINE ANAEROBIC CULTURES. *E. H. Spaulding* and *William Goode*, Department of Bacteriology and Immunology, Temple University School of Medicine, Philadelphia.

Continuing a study previously reported (J. Bact., 1938, 35, 54–55) an additional series of routine clinical specimens has been cultured on duplicate aerobic and anaerobic blood agar plates containing beef infusion base. Of 1358 specimens showing growth, 90 per cent were positive on both plates, 1.2 per cent on the aerobic plate only and 8.8 per cent on the anaerobic plate alone. Of 119 specimens containing anaerobes exclusively, 50 revealed hemolytic streptococci. In fact, 42.9 per cent of all the hemolytic streptococci isolated were recovered only by anaerobic culture. Nine per cent of the pneumococci and 25 per cent of non-hemolytic streptococci were, likewise, isolated as anaerobes. Thirteen of 28 cultures of *Hemophilus influenzae* were anaerobic upon primary isolation.

Most of the cultures mentioned above were temporarily anaerobic since sub-culture from the primary anaerobic plate produced satisfactory aerobic growth. Permanently anaerobic hemolytic streptococci, pneumococci and other organisms were occasionally recovered, however. The use of fluid or semi-solid media yielded results intermediate between those of the blood agar plates.

An observation of practical significance was the striking contrast between the aerobic and anaerobic growths of the facultative anaerobic hemolytic streptococci. Almost invariably the anaerobic growth was more rapid, more luxuriant and more hemolytic than the corresponding aerobic culture.

AN IMPROVED ANAEROBIC APPARATUS. *E. H. Spaulding*, Department of Bacteriology and Immunology, Temple University School of Medicine, Philadelphia, Pa.

A modification of the anaerobic apparatus described by Weiss and Spaulding (J. Lab. Clin. Med., 1937, 22, 726) was used in the study reviewed above. A satisfactory jar with an aluminum top was described. A vacuum gauge has been substituted for the mercury manometer and metal stop-cocks for the glass stop-cocks. The entire apparatus is housed in a wooden cabinet with a hard-rubber panel.

ONE HUNDRED AND THIRTY-SIXTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, FEBRUARY 28, 1939, PHILADELPHIA, PA.

NUCLEOPROTEINS FROM STREPTOCOCCUS PYOGENES: SOME CHEMICAL AND SEROLOGICAL PROPERTIES AND CHANGES IN BOTH CAUSED BY CERTAIN ENZYMES. *Charles A. Zittle*, Department of Bacteriology, University of Pennsylvania, School of Medicine, Philadelphia.

A nucleoprotein (M) has been extracted from whole organisms with N/20 HCl at 56 C., reacting type-specifically like Lancefield's "M". M was compared with a nucleoprotein (NPA) described previously in reports from this department.

M gave negative Millon's and Hopkins-Cole reactions; color tests were positive for ribose nucleic acid. NPA gave positive Millon's and Hopkins-Cole reactions and positive color tests for ribose and desoxyribose nucleic acid. The presence of ribose nucleic acid was confirmed by the specific action of ribonuclease.

Destruction of type-specificity of M by trypsin was confirmed and simultaneous loss of precipitability with acid found. This was rapid; 0.05 mg. of trypsin per ml. of a 0.2% solution destroyed acid precipitability within one minute and serological tests after three minutes showed specific precipitation also was destroyed. Alcohol precipitation of tryptic digests of M gave a high-phosphorous, biuret-negative component. A biuret-positive, low-phosphorous, non-diffusible component was obtained from the alcoholic solution.

Trypsin destroyed the precipitation of NPA with anti-NPA sera but not with anti-streptococcus sera. NPA precipitated at pH 1 but not at pH 3.5. Commercial trypsin destroyed precipitation at the lower pH. This was probably caused by the ribonuclease in commercial trypsin.

Some Observations on the Etiology of Vaginal Infections. *A. E. Rakoff*, Departments of Bacteriology and Obstetrics, Jefferson Medical College, Philadelphia, Pa.

Evidence was presented that the vaginal flora is largely dependent upon the presence or absence of estrogenic hormone. When present in sufficient concentration it causes growth of the vaginal epithelium and probably deposition of glycogen in the cells. Glycogen is partially utilized by a non-bacterial ferment, producing sufficient acidity to favor the presence of Döderlein's bacilli, which further ferment the available carbohydrate to produce a degree of acidity inimical to most bacteria (pH 4.0–4.8).

The absence of this mechanism, after the first week of life to puberty, leaves the vaginal tract of the child susceptible to various bacterial infections, both "non-specific" and gonorrheal. The same situation prevails after menopause.

Bacterial infections of the vaginal tract are rare during the sexually-mature period of life. Secondary bacterial infections are often attributable to infections of the cervix. Infestations with *Trichomonas vaginalis*, are very common. Data were presented supporting the belief that the protozoan parasite is associated with a distinct clinical entity, of which it is the primary etiologic factor, although it is always accompanied by a disturbance in the bacterial flora. The rôle of the accompanying bacteria was discussed, none of which were thought to be of primary etiologic significance, including the so called *"Streptococcus sub acidus"*.

The significance of monilia and leptothrix in the vaginal tract was also discussed.

The Action of Sulfanilamide on Hemolytic Streptococci in Human Blood and Serum. *John S. Lockwood and Helen M. Lynch*, Department of Research Surgery, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The population curve of young streptococci growing in normal human blood and serum, and the influence of sulfanilamide on this curve, has been determined. If the inoculum is washed free of peptone before being added to the serum, sulfanilamide shows a marked inhibiting effect on bacterial survival even in the absence of leukocytes. The character of the curve is subject to considerable variation, depending upon a number of factors, among them the size of inoculum, concentration of sulfanilamide, temperature of incubation, amount of free

hemoglobin. None of these factors is as significant in altering the effect of sulfanilamide as is the content of added peptone. Amounts of peptone as small as 1.0 milligram per 100 ml. will tend to inhibit the bacteriostatic, and particularly the bactericidal, effects of the drug in vitro. Streptococci which die out in sulfanilamide-serum tend to swell up and disintegrate, losing first their gram-positiveness. Streptococci which multiply slowly in serum containing sulfanilamide and peptone tend to form very long chains, and have the morphologic appearance of rough variants, though when transferred to drug-free media they rapidly regain their usual morphology. Phagocytosis appears to be effective in disposing of the organisms only under conditions of marked sulfanilamide bacteriostasis. Peptone inhibits the bacteriostatic action of sulfanilamide on *Escherichia coli* in urine, and of sulfapyridine on hemolytic streptococci and pneumococci in human serum. An explanation of the action of sulfanilamide which satisfies all of the observed phenomena is that the drug prevents the satisfaction of nutritional requirements of susceptible organisms in certain media. It seems probable that the drug interferes with the nitrogen requirements of bacteria when they are not supplied with an excess of assimilable amino-nitrogen.

One Hundred and Thirty-Seventh Meeting, Philadelphia County Medical Society Building, March 28, 1939, Philadelphia, Pa.

Hunting Tubercle Bacilli Fifty Years Ago. *Joseph McFarland*, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The method demonstrated was amusing and instructive, as exemplifying the improvements in the technic of clinical bacteriology, and was the one taught to medical students in the University of Pennsylvania from about 1885 to 1889. It was a modification of Koch's original method to accommodate it to medical students. The original Koch method required twenty-four hours staining at room temperature but the instructor applied greater heat so as to complete the procedure in the length of time at his disposal, usually an hour. The instructor possessed only the crudest of equipment and no oil immersion lens. The technic was somewhat as follows: to a three inch evaporating dish was added a saturated aqueous solution of gentian violet and 10% KOH solution added until the surface appeared metallic. The sputum was spread upon a cover-glass by means of a match stick, allowed to dry and fixed by passing through a flame. The fixed smear was floated film-side down on the surface of the prepared staining solution which was then heated to gentle steaming for 15 minutes by means of an alcohol lamp. The stained smear was then washed in 5% aqueous solution of vesuvin, washed in water and dried. Of one thing the instructor was sure: The detection of tubercle bacilli depended upon a color reaction and at the completion of the staining technic "whatever was blue was tubercle bacilli and nothing else, whatever was anything else could not be tubercle bacilli"! Of course the instructor might not believe that the tubercle bacilli were the cause of tuberculosis, and probably had never seen tubercle bacilli except when shown

to him by Koch or some other capable technician.

NOTES ON THE HISTORY OF PURE CULTURE METHODS. *J. R. Schramm,* University of Pennsylvania, Department of Botany, Philadelphia.

THE INTRODUCTION OF AGAR AGAR INTO BACTERIOLOGY. *A. P. Hitchens and Morris Leikind,* University of Pennsylvania, School of Medicine, Philadelphia, Pa. and Johns Hopkins University, Baltimore, Md. Published in J. Bact., 1939, 37, 485–493.

ONE HUNDRED AND THIRTY-EIGHTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, APRIL 25, 1939

A SYMPOSIUM ON LYMPHOPATHIA VENEREUM, THE SIXTH VENEREAL DISEASE, by members of the Proctologic Department of the Graduate Hospital, University of Pennsylvania, Philadelphia, Pennsylvania.

HISTORY, BACTERIOLOGY, FREI TEST AND ITS EVALUATION. *Harry E. Bacon and Francis D. Wolfe.*

GROSS LESIONS. *Collier F. Martin.*

PATHOLOGY. *Eugene Case and M. S. Hwang.*

LYMPHATICS. *Oscar V. Batson.*

BLOOD CHEMISTRY FINDINGS. *Charles A. Jones.*

ROENTGENOGRAPHIC INTERPRETATIONS OF RECTAL STRICTURE AND LYMPHOPATHIA VENEREUM. *Arthur Finkelstein.*

ONE HUNDRED AND THIRTY-NINTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, MAY 23, 1939, PHILADELPHIA, PA.

A METHOD FOR MAKING BACTERIAL COUNTS IN A TEST TUBE. *Edward Redowitz,* The Clinical Laboratory of Claude P. Brown, M.D., 1930 Chestnut St., Philadelphia, Pa.

A method was described for making counts in test tubes on non-motile bacteria, by employing semi-solid agar. The counts were made on *Lactobacillus acidophilus,* streptococcus and staphylococcus cultures. The colonies can be counted after 18 hours incubation as they are large and easily seen by transmitted artificial light.

The method is to use one set of tubes. Suitable medium containing 0.15% agar is introduced into the tubes with a sterile 10 ml. pipette in 9 ml. amounts. Then 1 ml. of the culture to be counted is transferred into a bottle containing 99 ml. of sterile saline solution and the pipette discarded. After a thorough shaking of the bottle, 1 ml. of the 10:00 dilution is pipetted into the first tube containing the semi-solid medium and thoroughly mixed. With the same pipette like transfers of 1 ml. are made successively into the remaining tubes. From the last tube 1 ml. is discarded after making the transfer. The tubes are incubated 18 to 14 hours and the colonies counted in those tubes where they are well separated. An average of two or three dilutions should be taken.

Since each tube contains only 9/10 ml. of the previous dilution then the formula is as follows: Number of colonies in tube × dilution multiplied by $\frac{10}{9}$ gives the number of bacteria in 1 ml. of culture.

THE PATHOGENESIS OF RHEUMATIC FEVER. *Mark P. Schultz*, National Institute of Health, Washington, D. C.

BACTERIAL ALLERGY. *Paul H. Langner*, Department of Allergy, University of Pennsylvania Hospital, Philadelphia, Pa.

A TYPHOID-LIKE INFECTION CAUSED BY A SLOW LACTOSE-FERMENTING ORGANISM. *John Eiman and Russell H. Fowler*, Abington Memorial Hospital, Abington, Pa.

A child was recovering satisfactorily from first and second degree burns when suddenly the clinical picture changed and death ensued.

At autopsy Peyer's patches were prominent. The tips of the villi were necrotic and floated off like flakes of rice. The spleen was enlarged and there was general lymph adenopathy. Lymph nodes showed marked proliferation of endothelial cells of the sinusoids and necrosis of germinal centers. Liver showed cloudy swelling, early fatty infiltration and numerous focal necroses. Lungs showed interstitial pneumonitis.

Pure cultures of gram-negative, non-spore-forming bacilli were isolated from the blood several days before death and from the ileum, Peyer's patches and colon at autopsy. These cultures fermented glucose, sucrose, maltose, mannite, dulcite, galactose, xylose, levulose and arabinose with acid and gas. Glucose, sucrose, arabinose and mannite showed a reversion of the pH after several days incubation. Lactose was fermented slowly, acid occurring on the third day, gas appearing on the 4th day. The organism is V. P. +, M. R. −, indole −, motile, gelatin liquefaction −, grows on Simmon's citrate agar and coagulates milk on the 7th day.

Serum from rabbits immunized with this organism failed to agglutinate two species of *Aerobacter*, six species of *Escherichia* and twelve species of *Salmonella*. It agglutinated paradysentery strains Flexner Y, Newcastle and Strong to approximately one-fourth titer. Absorption tests with these three strains failed to remove the agglutinins for the homologous strain.

STUDIES ON THE LIFE AND DEATH OF BACTERIA

I. The Senescent Phase in Aging Cultures and the Probable Mechanisms Involved

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The phenomenon of growth and death of bacteria in ordinary culture media has been studied in considerable detail. The data obtained in such studies show that growth follows a definite course, represented frequently by a curve constructed by plotting the logarithms of the numbers of organisms against time. This logarithmic growth curve has been divided into four main parts: the lag, logarithmic growth, maximum stationary, and death phases. After reaching the maximum stationary phase, the curve is frequently shown tapering off until it meets the abscissa, indicating sterility of the culture in a few days or weeks. However, a few of the more recent writers (Gay, 1936; Topley and Wilson, 1936) have made suppositions to the contrary. Gay states: "It is very probable that many cells may be unable to multiply but are still able to carry on other metabolic functions." He further suggests that "The number of viable organisms may also show repeated irregular increases possibly indicating spurts in multiplication. The long period of slow decline has not been studied as intensively as it probably deserves."

During the course of our investigations on factors affecting bacterial growth, certain observations suggested that a closer quantitative examination of death and death rates in bacterial cultures might be desirable. The period of prolonged survival and the probable mechanisms involved proved to be of particular interest.

I. THE SENESCENT PHASE

Methods

Sarcina lutea and *Serratia marcescens* were grown in flasks containing 1000 ml. of ordinary nutrient broth. The flasks were incubated at room temperature (approximately 24°C.), stoppered with cotton, and capped with lead foil to prevent excessive evaporation. The volume of the medium was kept constant by the periodic addition of sterile, double-distilled water. For a period of two years, duplicate plate counts of the number of cultivable bacteria were made at various intervals. During the 13th month of incubation, counts were made every other day for three weeks to determine any minor changes or fluctuations in the number of cultivable bacteria during this period.

Cultural characteristics

During the prolonged period of incubation the reaction of the aging cultures of *S. lutea* and *S. marcescens* became alkaline, reaching pH 8.49 and 8.12, respectively, as measured by the glass electrode. This is striking, especially in the case of *S. marcescens* whose limit of reaction is usually considered to be pH 8.0. When the reaction of one-year old *S. marcescens* cultures was adjusted to pH 7.0, the count rose in two weeks from 7,500,000 to slightly over a billion cells per ml., thus slowly approximating the number of cells found during the maximum stationary phase.

Microscopic examination of the aging cultures showed intact cells, a considerable amount of debris, and "ghost" forms. The cells were typical in shape and grouping and gave characteristic reactions to the gram stain. As is usually the case with dead cells, most of the cells of *S. lutea* stained gram-negative. The physiologic reactions of transplants of these cultures in litmus milk, gelatin, citrate medium, and in tryptophane, nitrate, and sugar broths remained constant. In the case of *S. marcescens*, there was definite evidence of variation when the cultures were plated out. The colonies ranged in color from white to uniformly deep red, and in morphology from the tiny, glistening to the spreader type.

That there are differences in resistance to adverse environmental conditions of cultures 3 to 6 hours old as compared with those 1 to 7 days old has been shown by Sherman and Albus (1923).. Hence it was thought desirable to determine roughly whether or not such a phenomenon had occurred in these aging cultures.

The methods of these investigators were followed in subjecting the two-year old cultures of *S. lutea* and *S. marcescens* to $HgCl_2$, phenol, and high temperatures. The action of $HgCl_2$ and of phenol was tested by allowing the organisms to remain in contact with the chemical for definite time intervals after which one loopful of this suspension was transferred to a tube of nutrient broth. If no growth occurred in the broth within 48 hours, it was concluded that the organisms had been killed by the chemical agent. Young eight-hour old cultures of these organisms were run as controls.

The results, recorded in table 1, accord in general with those reported by Sherman and Albus in that they indicate physiologic differences between young and old bacterial cells. A repetition of the experiments gave similar results.

Sherman and Albus also found that mature cells may be agglutinated by acid whereas young cells are not. Several rough determinations of this kind were made on the aging cultures of *S. marcescens* and *S. lutea*, but no differences were noted between the agglutinability of the two-year old cultures and those 8 hours old.

Population studies of aging cultures

Plate counts of these aging cultures showed the number of cultivable organisms to be remarkably high. Counts of from 5,000,000 to 30,000,000 bacteria per ml. were obtained during the thirteenth month of incubation. At the end of the two-year period the plate count of *S. lutea* was 1,000,000 bacteria per ml., while that of *S. marcescens* was 1,700,000 per ml.

Plate counts of the number of cultivable cells of *S. lutea* and *S. marcescens* plotted against time are shown logarithmically in figures 1 and 2. These two curves represent the numbers of cultivable bacteria in nutrient broth cultures maintained over a

period of two years. After an early initial drop which occurs during the first few days, the curves tend to level off and maintain an almost imperceptible decline. This gradual decline, however, is probably not one of total inactivity. Frequent counts, made

TABLE 1

Effect of adverse conditions

TIME	SERRATIA MARCESCENS		SARCINA LUTEA	
	2 years	*8 hours*	*2 years*	*8 hours*
	Exposure to 1 per cent phenol			
minutes				
0	+	+	+	+
1	+	+	+	+
2	+	+	+	−
5	+	−	+	−
10	−	−	+	−
15	−	−	−	−
20	−	−	−	−
	Exposure to $HgCl_2$ (1:12,000)			
0	+	+	+	+
1	+	+	+	+
2	+	−	+	+
5	−	−	+	+
15	−	−	+	+
30	−	−	+	+
45	−	−	+	+
60	−	−	+	+
120	−	−	−	−
	Exposure to 55°C.			
	Cells per ml.	Cells per ml.	Cells per ml.	Cells per ml.
0	1,700,000	8,000,000	1,000,000	950,000
5	90,000	1,000	980,000	15,000
15	30	0	400,000	75
30	0	0	80,000	10
45	0	0	600	0
60	0	0	0	0

during the twelfth and thirteenth months, revealed small rises and falls in the curve, thus indicating some evidence of actual multiplication and spurts of increase during the period. We have termed this period the "senescent phase."

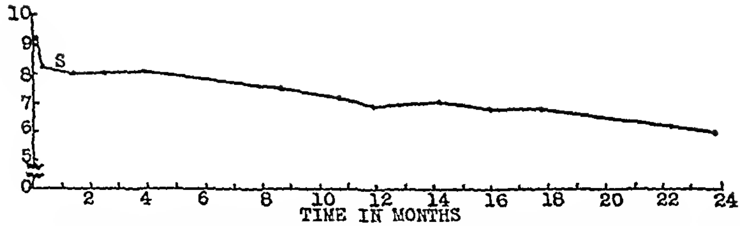

FIG. 1. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. LUTEA DURING TWO YEARS OF INCUBATION

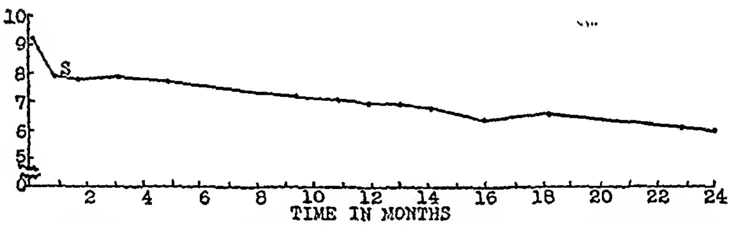

FIG. 2. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. MARCESCENS DURING TWO YEARS OF INCUBATION

"*S*" marks the beginning of the senescent phase. The early phases of the typical growth curve do not appear because of the time scale used.

TABLE 2

Representative counts of the numbers of bacteria per ml. in aging cultures

BACTERIUM	AVERAGE NUMBER OF BACTERIA PER ML.			AVERAGE REACTION OF CULTURE AT END OF ONE YEAR
	Immediately after inoculation	After 48 hours incubation	After one year incubation	
				pH
Sarcina lutea	6000	1,000,000,000	5,200,000	8.60
Serratia marcescens	8000	2,000,000,000	4,800,000	8.78
Bacillus subtilis	4000	10,000,000	300,000	8.92
Staphylococcus aureus	7000	1,800,000,000	1,300,000	8.62
*Staphylococcus aureus**	6000	2,000,000,000	750,000	8.61
Aerobacter aerogenes	5000	1,100,000,000	1,600,000	8.75
Eschcrichia coli	9000	1,700,000,000	300,000	8.91
Klebsiella pneumoniae	6000	2,500,000,000	775,000	8.81
Pseudomonas aeruginosa	4000	1,400,000,000	1,100,000	8.87
Rhodococcus rhodochrous	1000	700,000	430,000	7.86
Micrococcus tetragena	2000	300,000,000	2,100,000	8.83

* A non-proteolytic strain.

Table 2 gives data showing the partial results obtained in similar experiments using other bacteria for a period of one year. For the most part, the counts recorded represent an average of two to four separate cultures of each organism. The reaction of each culture at the end of this period is also indicated. Briefly stated, the results were similar to those obtained with the aging cultures of *S. lutea* and *S. marcescens*. In each case, after the early initial drop, the curve tends to level off in a manner similar to that already described for *S. lutea* and *S. marcescens*. In general, when the aging cultures were plated out, the resulting growth was very slow, thus requiring incubation periods longer than is ordinarily necessary for these species. The number of bacteria at the end of the one-year period is given in the table because in all cases this was the lowest number of bacteria per ml. at any time during the 12-months period.

II. STUDIES ON THE PROBABLE MECHANISMS INVOLVED

Speculation as to why bacterial populations are maintained over a long period of time brought up the question of whether a bacterial species could utilize cells of the same or of other species as a source of nutriment. It was not thought that this would be the sole explanation for the continued growth in the aging cultures, but it seemed desirable to know to what extent it might play a part. Consequently, experiments were conducted to test whether bacteria could utilize other bacterial cells as the only source of food.

Methods

For this purpose, pure cultures were grown on nutrient agar in 1000 ml. Blake bottles. The cells were harvested and then washed three or four times by suspending them in physiologic saline and centrifuging. An estimated 1 per cent suspension of moist cells in 1.5 per cent washed granulated agar was adjusted to pH 7.0 and autoclaved for 20 to 30 minutes. The washed cells plus washed granulated agar constituted the test medium, the only source of nutrients being the bacterial cells incorporated in the agar. Plates of this medium were then

poured in the ordinary manner and streaked with the various test organisms. Washed agar alone and ordinary nutrient agar were used as controls. Forty-eight hours after inoculation, the growth on the cell-agar medium was compared with the growth on the nutrient agar plates.

The cell-agar media used were made of the whole cells of *S. marcescens*, *S. lutea*, *Staphylococcus aureus*, and the intact as well as the disrupted cells of *Escherichia coli*. The disruption of the cells was brought about by repeated freezing and thawing of the bacterial suspension.

Results

In general, as can be seen from table 3, the organisms that are proteolytic in action grew best on the cell-agar medium. Out of twenty-one species of organisms tested, best growth was obtained with *S. lutea*, *S. marcescens*, *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus anthracis*, and molds of the genera *Penicillium*, *Aspergillus*, and *Mucor*.

Partial but not normal growth was obtained with *E. coli* on *E. coli* cells. It seemed to make no difference whether these cells were intact or disrupted. Partial growth was also obtained with *Staphylococcus aureus* on *S. lutea* cells, *Aerobacter aerogenes* on *E. coli*, and *Pseudomonas aeruginosa* on *S. lutea* and on *S. marcescens*. *Corynebacterium diphtheriae* gave partial growth on *E. coli*, while *Klebsiella pneumoniae* gave light growth on all the test media.

No appreciable growth was obtained on any of the test media with the following organisms: *Leuconostoc mesenteroides*, *Neisseria catarrhalis*, *Rhodococcus rhodochrous*, *Micrococcus tetragena*, *Streptococcus viridans*, and *Streptococcus hemolyticus*.

Relationship to proteolysis

One of the most striking reactions observed was the marked digestion of the cells in the medium by some of the species streaked upon its surface. This was particularly noticeable on the cell-agar media inoculated with *S. lutea* and *B. subtilis*. These species digested the cells in the otherwise opaque media to such

an extent that distinct, clear zones could be seen about the growing colonies. *S. marcescens* showed much smaller, although quite definite, zones of digestion. Blocks of digested and undigested areas of the cell-agar medium were removed, embedded

TABLE 3

Growth of organisms on cell-agar medium

INOCULUM	SUBSTRATE						
	S. lutea	*S. marcescens*	*S. aureus*	*E. coli*	*E. coli* (disrupted cells)	Control (nutrient agar)	Control (agar alone)
Sarcina lutea	++++	++++	+	+++	+++	++++	−
Serratia marcescens	++++	++++	−	+++	+++	++++	−
Bacillus subtilis	++++	++++	−	+++	++	++++	−
Bacillus mycoides	++++	++++	+	++++	++++	++++	−
Bacillus anthracis	+++	+++	−	++	++	++++	−
Aerobacter aerogenes	−	−	−	+	+	++++	−
Escherichia coli	+	++	−	+	+	++++	−
Klebsiella pneumoniae	++	+	−	+	+	++++	−
Pseudomonas aeruginosa	++	+	−	−	−	++++	−
Leuconostoc mesenteroides	−	−	−	−	−	+	−
Neisseria catarrhalis	+	−	−	+	−	++++	−
Rhodococcus rhodochrous	−	−	−	−	−	++	−
Micrococcus tetragena	−	−	−	+	+	++++	−
Corynebacterium diphtheriae	−	−	−	++	+	+	−
Staphylococcus aureus	++	−	−	++	+	++++	−
*Staphylococcus aureus**	+	−	−	+	−	++++	−
Streptococcus hemolyticus	−	−	−	−	−	−	−
Streptococcus viridans	−	−	−	−	−	−	−
Aspergillus sp	+++	+++	++	++++	++++	++++	−
Penicillium sp	++++	+++	++	++++	++++	++++	−
Mucor sp	++++	+++	++	++++	++++	++++	−

* A non-proteolytic strain.

in paraffin, sectioned, stained and examined. In the undigested part, the bacteria were found intact and in their characteristic groupings, while in the digested part there were fewer bacteria and those that were present appeared to be in the process of disintegration.

Additional reactions

S. marcescens, which rarely produces a metallic sheen when grown on nutrient agar, produced a very pronounced sheen when grown on its own cells. It was also observed that although *S. marcescens* produced no pigment when grown on the whole cells of *E. coli,* it again produced a deep red pigment when transferred to nutrient agar.

E. coli grew very meagerly on its own cells. However, when a sugar such as lactose was added, normal growth resulted. If the medium was made of cells of *E. coli* previously cultured on a nutrient agar medium rich in lactose it supported the growth of *E. coli* no better than did the medium made of cells propagated on ordinary nutrient agar.

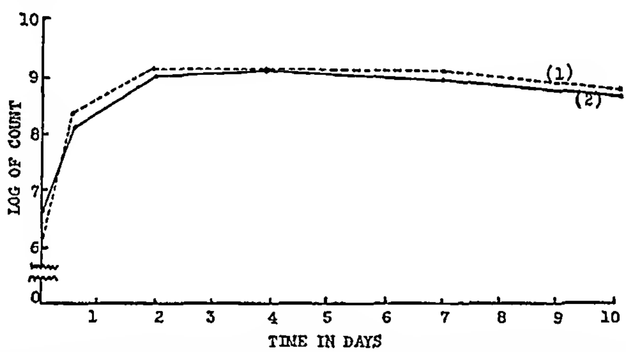

FIG. 3. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. MARCESCENS GROWING IN ORDINARY NUTRIENT BROTH (1) AND IN A WASHED SUSPENSION OF S. MARCESCENS CELLS (2)

Cell suspension medium

When washed, 1 per cent suspensions of autoclaved cells of *S. marcescens* and of *S. lutea* were suspended in saline and inoculated with the homologous organisms, normal growth resulted (fig. 3). Plate counts of this growth corresponded to the results obtained on the cell-agar plates. It is interesting to note that in the case of *S. marcescens* there still remained over 40,000,000 viable bacteria per ml. in the suspension, even after three months incubation.

Although these experiments indicate that not all bacteria grow

upon the cells of the same or different species, some bacteria seem to have a marked ability to use bacterial cells as a source of nutriment. No doubt these experiments illustrate only a few of many such "cannibalistic" relationships between bacterial species. This study is, therefore, being broadened to include any such possible relationships between the more fastidious bacteria.

DISCUSSION

It seems that most investigators consider that bacteria in nutrient broth cultures die or decrease in numbers in a manner similar to and governed by the same laws which determine the manner or rate at which they die when subjected to such unfavorable environments as disinfectants. Apparently little or no effort has been made to learn the fate of the culture after the rapid drop in numbers following the *maximum stationary phase.* Most workers seem to have concluded that the decline continues quite steadily until the culture reaches sterility. This may be the case when bacteria are grown in media containing a fermentable sugar or when bacteria are acted upon by strong disinfectants.

The results of this investigation indicate that the period of death is not necessarily one of regular decrease when bacterial cultures are grown in ordinary nutrient broth. This is evident from the small rises and falls in the curve which probably indicate spurts in multiplication. In general, however, the curve secured when the logarithms of the numbers of bacteria are plotted against time shows a slow, almost unnoticeable decline. With such data as evidence, it seems that we are here presented with a period in the life of bacterial cultures which has not had its due share of recognition. We consider it a distinct phase in the culture cycle of bacteria and suggest that it be designated as the "senescent phase." This is in accord with the terminology used in connection with the other parts of the growth curve. Whether all bacteria possess this phase will require further investigation.

Any discussion as to how the senescent phase is maintained

would, as yet, have to be more theoretical than factual. The maze of interrelated factors makes the solution particularly difficult. In accord with general conceptions, the criterion of death in such experiments as these will, of necessity, have to be the inability of cells to reproduce when placed in a favorable environment, as, for instance, when transferred to agar plates for the purpose of counting. It is, furthermore, possible that cells may be able to carry on other metabolic functions but be unable to reproduce. Then too, the "resting" stage, studied by Quastel (1926) and Kendall (1930), may be an important factor.

Regardless of the attacks made against Chick's (1908) theory of variable resistance of individual cells in regard to the death rates of bacteria as influenced by disinfectants, such a possibility must also be considered. This is likewise suggested by what Sherman and Albus (1923) have termed "physiological youth." According to these authors the physiologically young bacteria are more sensitive to adverse conditions than the old. As the cultures age, the remaining cells may become old and are thus able to withstand the adverse conditions of alkaline reaction, accumulation of waste products, and other detrimental environmental conditions which ordinarily kill those organisms which are physiologically young. The possibilities that the cultures are undergoing variation and that the medium is acting as a selective agent must also be considered. This may partially explain the occasional spurts in multiplication during the senescent phase.

Although perhaps of less significance than originally supposed, many writers still consider starvation to be one of the causes of death of bacteria in old cultures. Therefore, we were prompted to ask ourselves if the dead organisms in aging cultures could be of sufficient nutritive value to serve as a source of necessary food elements to aid in maintaining the senescent phase. One can readily imagine that due to long standing the dead cells remaining in the culture autolyze and liberate some nutriment which is used by the remaining viable cells. It might further be supposed that dead bacterial cells contain all the constituents necessary for the growth of new cells of the same species pro-

viding, of course, that the living cells possess enzymes necessary to utilize the dead cells. The data here presented indicate the possibility of such a cycle. Just how many dead cells are needed to supply the energy for the growth of one living cell could only be determined by more accurate analytical methods. It is no doubt true that a 1 per cent suspension of bacteria autoclaved at their height of development is not comparable with the cells of very old cultures. Nevertheless, the results secured in growing *S. marcescens* in a saline suspension of killed cells of the same species would seem to have particular significance in this respect. This medium, in which the only source of food was the dead bacterial cells, supported growth to a degree almost as great as nutrient broth. For the want of a better word this relationship has been referred to as "cannibalism," a term used by Kollath (1924) in reference to a similar phenomenon. This phase of the work is being further investigated.

SUMMARY

Periodic plate counts made of aging broth cultures of *Sarcina lutea* and *Serratia marcescens* showed the numbers of cultivable bacteria remaining in the cultures to be remarkably high even after two years incubation. Similar findings have been obtained with a number of other organisms after one year of incubation. Readjusting the reaction of the cultures of *S. marcescens* from pH 9.0 to 7.0 gave a definite increase in the number of viable organisms.

Microscopic examination of these aging cultures did not show much cellular variation, although on plating colonial variation was evident in some instances, especially with *S. marcescens*. The species retained their physiologic and gram stain characteristics. Rough determinations indicated that the aging cells possessed a marked resistance to adverse environmental conditions.

When the counts were plotted logarithmically, the resulting curve, after the initial drop following the maximum stationary phase, tended to level off and maintain an almost imperceptible decline. Small rises and falls in the curve were still apparent

during this period. The term "senescent phase" is suggested for this period of the curve.

Varying degrees of growth were obtained with 21 strains of organisms on media in which the only source of nutriment was a large quantity of washed and autoclaved bacterial cells. In general, the proteolytic organisms grew more luxuriantly on such media than did the non-proteolytic organisms.

When *S. marcescens* was inoculated into a liquid medium consisting of a suspension of killed cells of *S. marcescens*, the counts obtained were almost as great as those obtained in the nutrient broth controls.

The term "cannibalism" is used to designate the growth obtained in cultures where the dead cells are serving as a source of food.

The authors wish to acknowledge the valuable suggestions and criticisms offered by Dr. N. Paul Hudson in the preparation of this paper.

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A CULTURAL STUDY OF FILAMENTOUS BACTERIA OBTAINED FROM THE HUMAN MOUTH[1]

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In a survey of the literature dealing with "filamentous" oral bacteria (Bibby, 1935), it was found that the successful growth of such organisms has been reported on fifty-seven occasions. Most of the cultivated forms have been inadequately described; furthermore, no two descriptions are exactly alike. It is possible, nevertheless, to recognize fourteen as *Leptotrichia*, of which there are at least two recorded types, and seven as *Actinomyces*. Of the latter group, none has been sufficiently well studied to establish a filamentous morphology nor an origin in the mouth. Accordingly, a systematic study of oral filamentous bacteria was undertaken. The present report is a record of the cultural findings.

METHODS

The material for study consisted of "materia alba," calculus, and scrapings made from the teeth, gingivae and mucous surfaces at various sites in healthy and diseased mouths of adults and children. Specimens were collected on sterile dental instruments with precautions to reduce contamination to a minimum. Inoculations of various culture media were made immediately after collection.

[1] Based on portions of a thesis presented on October 29, 1935, by Basil G. Bibby to the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The study upon which this report is based was supported in part by grants from the Rockefeller Foundation and the Carnegie Corporation of New York.

In addition to Douglas' agar, rabbit-blood agar, and meat-extract agar, we employed the special media used by previous investigators. These included various enriched media, media made selective by the addition of inhibitory substances, and media of low nutritive value designed to favour the growth of fungus-like organisms. Incubation at 37°C. was carried out aerobically, and anaerobically, using Brown's (1922) modification of M'Intosh and Fildes' anaerobic method. To favour the development of fungus-like organisms, a few cultures were made at room temperature. For certain purposes a bent glass rod was employed to inoculate solid media. Early subcultures were made on the media which had been used in the original isolation, but thereafter, where growth was satisfactory, stock media were substituted.

Isolations were made under a binocular microscope at a magnification of 14 diameters. The separation of colonies was greatly facilitated by the use of a sharp-edged platinum spade about 1.5 mm. wide and 0.2 mm. thick.

Ascitic agar slopes containing 2 per cent of carbohydrate were used for fermentation tests. The acidity produced was estimated colorimetrically in 10-day cultures in Douglas' broth containing 5 per cent of glucose. Tests for diastatic activity were made in 0.3 per cent starch plates. Peptone water and Douglas' broth were used for determining the formation of indol.

CULTURAL OBSERVATIONS

Using morphology and colonial form as a basis for classification, it was possible to recognize a number of distinct groups of filamentous, pleomorphic, non-spore-forming, non-motile organisms. These groups are described below.

Group I

Organisms of Group I were found regularly in anaerobic cultures, in which they comprised from 0.5 to 2 per cent of the colonies. The individual elements were from 3 or 4 to 100 or more μ long and about 1 μ wide. Thread forms predominated in both liquid and solid media. The filaments were unsegmented.

straight or curved and sometimes intertwined in tangled masses. Ends were square or rounded, commonly resembling a thick bacillus from which a filament appeared to grow (fig. 1). Irregular elements occurred (fig. 2). Short and round forms were most prominent in smooth colonies; branched, forked, swollen, and irregular elements in intermediate colonies; and filamentous square-ended forms in rhizoid colonies. Young elements were uniformly gram-positive. In old cultures, irregular staining gave a coarsely granular or banded appearance. Completely decolorized "shadow" forms were not uncommon. The Ljubinsky stain showed round granules in all elements (fig. 3). Iodin did not give the blue "granulose" reaction.

Colonies of freshly isolated strains appeared as greyish white pin points after forty-eight hours on blood agar. These reached a size of 1 or 2 mm. in from 4 to 7 days. Mature colonies were circular, having a slightly raised smooth center and a flat peripheral portion, across which tangled filamentous processes radiated to give a rhizoid appearance (fig. 4). The colonies were tough and cartilaginous and emulsification was impossible. Ascitic and Douglas' agar, but not meat-extract agar, gave satisfactory growths. In subculture, atypical colonies often appeared (fig. 5), especially when cultivation was aerobic or when platings were made from old Douglas' broth cultures. A study of four strains during thirty "generations" showed that, although all strains did not react in quite the same way, anaerobic conditions and subculture on alkaline media tended to stabilize the rhizoid colonial type, whereas aerobic cultivation and the use of liquid media tended to produce smooth and unstable intermediate variants. In Douglas' broth, growth was slow, a scanty granular sediment appearing after a week. The granules were from 0.25 to 2 mm. in diameter and settled to the bottom or adhered firmly to the walls of the tube, leaving the medium clear. In subcultured strains, a heavy finely-granular growth often occurred.

Subcultures grew readily under atmospheric conditions. Slow growth took place at room temperature. A pH of from 7.0 to 7.3 was optimal, but growth occurred between pH 6.0 and 8.5. The organisms remained viable for four months in the incubator

or refrigerator, but did not survive drying in air. They were killed in five minutes at 65°C.

With a few exceptions, the fifty-three strains tested produced acid, but no gas, from glucose, levulose, maltose, sucrose, raffinose, and dextrin after from two to six days' aerobic or anaerobic incubation. Lactose, galactose, inulin, dulcitol, and mannitol were not attacked. Milk was not changed. Seven of thirty-four strains hydrolysed starch. The average pH produced by thirty-five strains after twelve days' growth was 5.1. On several occasions, the final reaction was pH 4.8. Gelatin and blood serum were not liquefied. Indol, ammonia, or hydrogen sulphide were not formed. Nitrates were reduced to nitrites. A comparison of seven rough and smooth strains did not reveal significant differences in the rate of acid-production or the final acidity attained. Injections of freshly isolated and of subcultured rough and smooth strains into guinea pigs, rabbits, and mice failed to produce any progressive pathological reaction.

Group II

Filaments of Group II showed some resemblance to those of Group I. They were observed in only a few cultures. But three strains were successfully subcultured. Individual organisms varied in length from 2 to 200 μ and in width from 0.8 to 1.8 μ (fig. 6). The filaments were generally twisted and sometimes showed septation. Branching occurred frequently. The ends were blunt or rounded and terminal clubbing was seen on a few occasions. The reaction to the gram stain was positive, but some threads decolorized completely and others only in parts. The Ljubinsky stain gave a granular appearance like the organisms of Group I. The iodin stain was negative.

After twenty-four hours, colonies were greyish white and from 1 to 5 mm. in diameter. The small colonies were convex, the larger ones (fig. 7) raised, with an even rounded edge from which extended fine filamentous outgrowths. The surface was finely granular or fibrous. The consistency was soft and emulsification easy. Sometimes "daughter" colonies with a smooth surface and even outline appeared. Growth occurred on blood,

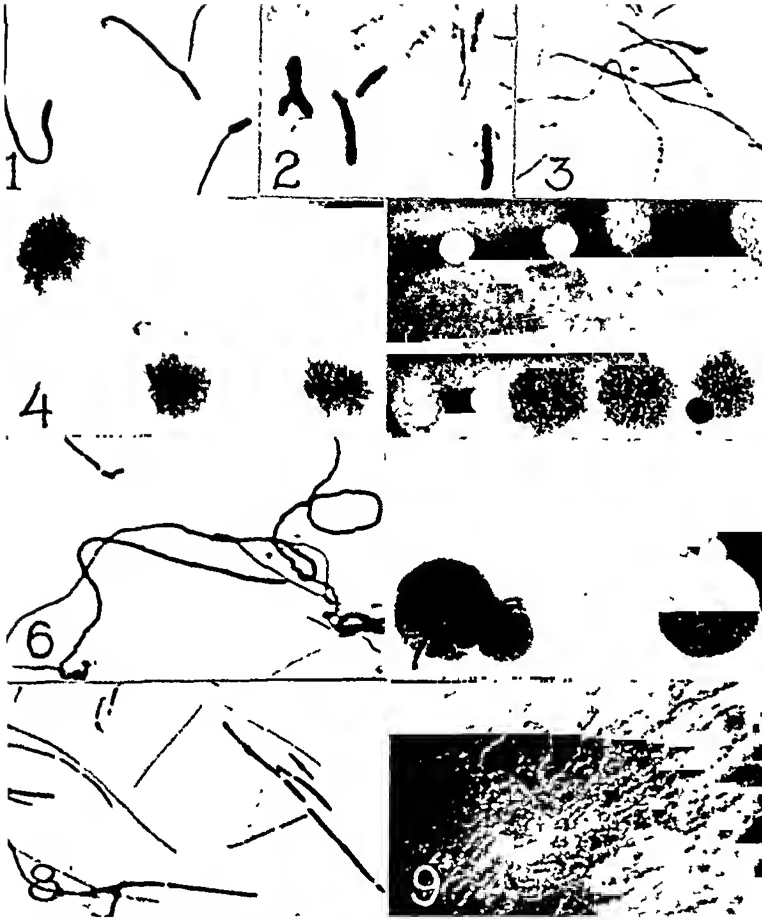

FIG. 1. SMEAR FROM 3-DAY BLOOD AGAR CULTURE, SHOWING FILAMENTOUS ELEMENTS, SOME ENDING IN CLUBS ("WHIP" FORMS)
Gram stain. × 1000

FIG. 2. PLEOMORPHIC ORGANISMS OF A SMOOTH VARIANT STRAIN FROM A 12-DAY DOUGLAS' BROTH CULTURE
Note degenerated forms. Gram stain. × 1000.

FIG. 3. SMEAR FROM 3-DAY BLOOD AGAR CULTURE SHOWING GRANULAR STAINING WITH LJUBINSKY STAIN. × 1000

FIG. 4. TYPICAL RHIZOID COLONIES OF 5-DAY CULTURE ON BLOOD AGAR. × 17

FIG. 5. COLONIAL VARIANTS IN A 6-DAY CULTURE ON BLOOD AGAR
The rough colonies are slightly atypical "B" forms and the smooth colonies are typical "S" types. × 17.

FIG. 6. SMEAR FROM 3-DAY BLOOD AGAR CULTURE
Note irregular staining. Gram stain. × 1000.

FIG. 7. COLONIES OF 3-DAY CULTURE ON BLOOD AGAR
Smooth "daughter" colonies are visible. × 20

FIG. 8. SMEAR FROM 6-DAY BLOOD AGAR CULTURE
Gram stain. × 1000

FIG. 9. COLONIES OF 6-DAY CULTURE ON HEMOLYZED-BLOOD ASCITIC AGAR. × 20

ascitic, Douglas' and meat-extract agars. In Douglas' broth, a slight stringy sediment was formed.

Good growth was observed under both aerobic and anaerobic conditions. Biochemical tests were completed with only two strains. One of these was actively saccharolytic, fermenting glucose, levulose, maltose, sucrose, lactose, and mannitol without the production of gas. Inulin was not fermented. The other did not ferment any sugar tested. Neither strain changed starch or milk. Gelatin and blood serum were not liquefied nor were indol or hydrogen sulphide produced. Both strains reduced nitrates.

Group III

The organisms of Group III (fig. 8) were straight or slightly curved rods and filaments from 5 to 100 μ long and from 0.7 to 1.0 μ thick. The ends were blunt, but occasionally rounded or tapered. Irregular swollen forms were encountered. The reaction to the gram stain was uncertain, most elements being completely decolorized. The Ljubinsky and iodin stains were sometimes weakly positive. After from seven to ten days, mature colonies (fig. 9) were dull grey or colorless, rough, slightly moist and irregularly shaped, having a slight central eminence from which interlacing ridges radiated to extremely filamentous edges. The colonies were adherent, but gelatinous and easily emulsified. Except in first transplants, growth occurred only on media containing blood and under strictly anaerobic conditions. In enriched Douglas' broth, a white coherent membranous sediment appeared which on shaking formed stringy floccules. Three strains produced acid without gas from glucose, maltose, levulose, and sucrose: one strain also fermented lactose, galactose, inulin, and raffinose and produced a pH of 4.2. Gelatin and blood serum were not liquefied. Nitrites and hydrogen sulphide were not produced. Indol was formed.

Group IV

The organisms of Group IV were from 7 to 50 μ long and between 0.7 and 1 μ broad. The ends were generally pointed.

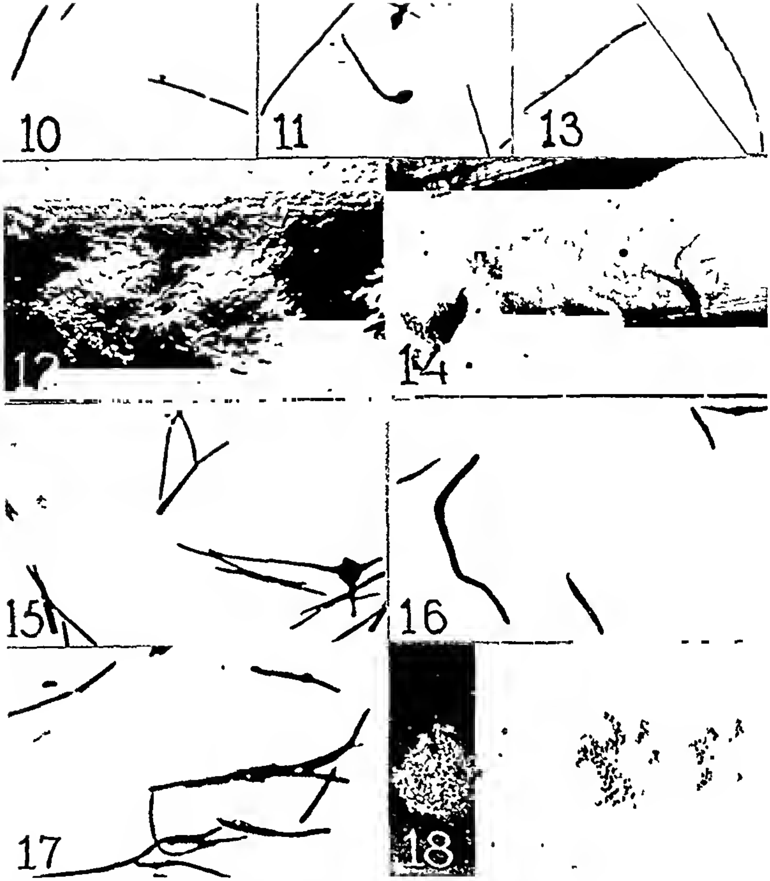

FIG. 10. SMEAR FROM 6-DAY BLOOD AGAR CULTURE, SHOWING BOTH FUSIFORM AND FILAMENTOUS ELEMENTS
Gram stain. × 1000

FIG. 11. SMEAR FROM 6-DAY BLOOD AGAR CULTURE, SHOWING IRREGULARLY SWOLLEN AND CLUBBED FORMS
Gram stain. × 1000

FIG. 12. COLONIES OF 6-DAY CULTURE ON BLOOD AGAR. × 20

FIG. 13. SMEAR FROM 6-DAY BLOOD AGAR CULTURE
Gram stain. × 1000

FIG. 14. COLONIES OF 6-DAY CULTURE ON GENTIAN-VIOLET BLOOD AGAR
This form of colony was found in original cultures. × 20

FIG. 15. SMEAR FROM 6-DAY BLOOD AGAR CULTURE, SHOWING FILAMENTOUS AND SWOLLEN ELEMENTS
Note definite branching. Gram stain. × 1400

FIG. 16. SMEAR FROM 6-DAY BLOOD AGAR CULTURE OF FILAMENTOUS ORGANISMS, SHOWING IRREGULAR, BENT AND SWOLLEN ELEMENTS
Gram stain. × 1000

FIG. 17. SMEAR FROM 6-DAY BLOOD AGAR CULTURE, SHOWING FILAMENTOUS AND SWOLLEN ELEMENTS
Gram stain. × 1400

FIG. 18. COLONIES OF 6-DAY CULTURE ON BLOOD AGAR FROM WHICH THE ORGANISMS SHOWN IN FIG. 17 WERE DERIVED. × 20

but when in tandem formations, square ends occurred (fig. 10). Sometimes one end was swollen and the other pointed. Swellings reaching a diameter of 2.5 μ were found (fig. 11). The reaction to the gram stain was positive, but when grown on gentian-violet media (used for isolation), the organisms retained the gram stain but slightly. The Ljubinsky stain revealed no granules. A positive iodin reaction occasionally occurred in organisms grown on media containing sugar. After five days on blood agar, colonies were 1 or 1.5 mm. in diameter, colorless, umbonate, with an uneven surface and small tangled ridges running to the edge, where they formed uneven outgrowths (fig. 12). The colonies were soft and emulsified easily. Growth was good on ascitic and Douglas' agar. In Douglas' broth, a slight clouding of the medium developed after two days and a greyish-white sediment was formed. Growth was most satisfactory under anaerobic conditions, although it sometimes took place under low oxygen tensions. Acid was produced from glucose, maltose, sucrose, and dextrin by the sixteen strains tested and only one failed to ferment levulose and lactose. Inulin, mannitol, and dulcitol were broken down by a few strains. Acid was produced in milk without clotting. Only a single strain of the six tested had diastatic properties. Of the variable acidities produced, pH 4.5 was the lowest. Gelatin and blood serum were not liquefied, indol and hydrogen sulphide were not formed, and nitrates were not reduced. The single strain tested was killed in five minutes at 65°C. Several strains survived storage in the incubator for five months.

Group V

Organisms of Group V (fig. 13), of which only a few strains survived subculture, were from 15 to 50 μ in length and about 0.8 μ in diameter, the longer forms occurring in subcultures. They were straight or curved, with blunt, rounded, and occasionally tapered ends. The reaction to the gram stain was positive except in old and "gentian-violet" cultures. The Ljubinsky and iodin stains were negative. The original colonies on gentian-violet blood agar were less than 1 mm. in diameter, having

inconstant, leaf-like, or ameboid shapes (fig. 14). They were colorless with a stippled surface and a butyrous consistency. Subcultured colonies generally had long rhizoid outgrowths and diameters of up to 8 mm. In Douglas' broth, growth was indefinite. A slight flocculent precipitate sometimes appeared. The organisms were strict anaerobes. None of three strains yielding satisfactory growth fermented carbohydrates and only a single strain reduced nitrates.

Group VI

A sixth group of organisms was clearly set apart by colonial type, morphology, and staining reaction. These organisms have been described elsewhere (Knighton and Bibby, 1933) and identified as *Fusiformis polymorphus* (Hine and Berry, 1937). There is no need for further mention here.

Group VII

An indefinite seventh group of filaments was created to embrace heterogeneous types which did not fit in any of the other groups. These organisms, of which only single strains were found, showed variable colonial form, morphology (figs. 15 to 17) and biochemical properties. Until more strains can be studied, detailed descriptions will serve no useful end.

DISCUSSION

The principal interest of the present work lies, perhaps, in the fact that it establishes the existence of a considerable variety of filamentous bacteria in the human mouth and indicates that there are further types which have not yet been cultivated. The groups recognized can be correlated with certain morphological types easily distinguishable in smears prepared directly from the mouth (Bibby and Berry, 1939) and with various organisms described by previous investigators. Table 1 presents a summary of these relationships.

Of the genera *Leptotrichia*, *Fusiformis*, or *Actinomyces* to which the oral filaments might be assigned, Groups I, II, III, and V seem to be acceptable as *Leptotrichia*, if Bergey's (1934) definition

be accepted. Since it is only in Group I that the constituent strains show uniform cultural and biochemical properties, the suggestion of a specific name is warranted for this single group only. For these organisms, we propose the name *Leptotrichia buccalis*, making this recommendation in full cognizance of Bergey's use of the name for another species. This suggestion is made because, as has been pointed out elsewhere (Bibby, 1935), we consider the organisms of the species in question, *Leptotrichia buccalis* (Robin) Trevisan, to be bacilli which have no place in the genus *Leptotrichia*.

TABLE 1

Relationships between the cultivated mouth filaments reported herewith (Groups I to VI), morphologically identified filaments, and previously cultivated filamentous oral bacteria

CULTURAL GROUP REPORTED HEREWITH	CORRESPONDING MORPHOLOGICAL TYPE (BIBBY AND BERRY, 1939)	SIMILAR ORGANISMS CULTIVATED PREVIOUSLY
I	I	*Leptothrix* (Meunier and Bertherand, 1898); *Leptothrix buccalis* (Kligler, 1915)*; (Bulleid, 1924)†; (Grythe, 1933); *Cladothrix matruchoti* (Mendel, 1919); *Streptothrix interproximalis* (Fennel, 1918)‡; *Actinomyces* "B1" and "C1" (Naeslund, 1925); *Leptotrichia buccalis* (Howitt and Fleming, 1930)
II		
III	II	
IV	III	*Leptothrix buccalis* (Brailovsky-Lounkevitch, 1915)†, ‡; *Leptothrix innominata* (Wherry and Oliver, 1916)†, ‡; *Leptothrix* (Jay, 1927)†, ‡; *Fusiformis dentium* (Bergey, 1934)*
V	III	
VI	IV	*Fusiformis polymorphus* (Bergey, 1934)

* Growth characteristics different from those reported herewith.
† Biochemical characteristics different from those reported herewith.
‡ Morphological characteristics different from those reported herewith.

The classification of the organisms in Group IV presents another difficult problem. Their properties embrace some of those considered to be characteristic of both the genera *Leptotrichia* and *Fusiformis*. Because present systems of classification offer no significant points of distinction between these genera

(although they are assigned to different bacterial families, *Actinomycetaceae* and *Mycobacteriacae* respectively), there seem to be no cogent reasons for preferring either genus.

The failure to find a significant difference between the occurrence of filaments in healthy and diseased mouths suggests that these organisms are not important in oral disease. The high concentration of acid formed by some of the strains in carbohydrate-containing media, however, supports the possibility that these filaments may have an important part in the causation of dental caries (Kligler, 1915). Furthermore, the adherent properties of the colonies of the organisms in Group I, which fix them tenaciously to the side walls of a glass culture-tube, points to the possible importance (Bulleid, 1924, Bibby, 1935) of this type in the formation of salivary calculus. The characteristic arrangement of filaments on the surfaces of the teeth suggests that they may exert a determining influence on the flora there. Thus the possibility of their playing an indirect but significant rôle in the mouth, by determining the basic nature of the oral flora as a whole, cannot be disregarded.

SUMMARY

By the use of anaerobic methods and of a wide variety of culture media, a large number of strains of filamentous bacteria have been isolated from the mouth. On the basis of a study of the morphology and the characteristics of growth of the eighty-three strains which were successfully carried in subculture, a tentative working division into seven groups is proposed. Of these, six groups have distinct characteristics. Only two, however, can be identified with organisms which have been isolated by previous investigators, although organisms showing some resemblance to a third group have been described. Four of the groups of filaments have the characteristics of the genus *Leptotrichia*, one those of the genus *Fusiformis*, and one those of both genera. Of all the groups, only one embraced strains all of which were alike in biological properties. For these organisms we suggest the name *Leptotrichia buccalis* because they represent the most commonly isolated of the culturable oral filaments.

ACKNOWLEDGMENT

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OPTIMUM TEMPERATURE FOR DIFFERENTIATION OF ESCHERICHIA COLI FROM OTHER COLIFORM BACTERIA

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During the past few years, several investigators have advocated the use of temperatures lower than 46°C. in the Eijkman test for *Escherichia coli.* Levine, Epstein and Vaughn (1934) suggested a temperature of 43° to 44°C. as optimum for selective growth of *Escherichia coli* in both the original Eijkman medium and in Standard Methods lactose broth. Their *Aerobacter* strains were markedly inhibited in both media at this temperature while some of the *Citrobacter* strains showed gas in the original Eijkman medium. None of the *Citrobacter* strains, however, produced gas at these temperatures in Standard Methods lactose broth of the A.P.H.A.

Skinner and Brown (1934) criticized the Eijkman test because strains of *Escherichia coli* failed to grow in Eijkman's or Bulir's medium if the tubes were placed directly in a water-bath at 46°C.

Wilson (1935), using the MacConkey medium, observed that practically all indol-positive strains of *Escherichia coli* grew well and produced gas from lactose at 44°C. None of his strains of *Aerobacter aerogenes* produced gas at this temperature and very few grew at all.

Minkevich, Alexandrov and Sobelova (1936) reported that a temperature of 46°C. did not hinder their *Escherichia coli* cultures from fermenting mannitol in Bulir's medium if inoculated heavily, but in many cases, they contended, the high temperature of 46°C. repressed growth of small numbers. Therefore, they proposed to make fermentation tests in Bulir's medium at 43° to 43.5°C.

Dodgson (1938) who has studied the application of the coliform index of pollution to shellfish for a number of years has concluded

"there can be no legitimate standard of purity for purified shellfish based on tests carried out at a temperature of 37°C." The practical limitations of using 37°C. for isolation of *Escherichia coli* from shellfish have been demonstrated time and again by extensive data on this subject by Perry and Hajna. Dodgson uses MacConkey's lactose bile medium at a temperature of 44°C.

The authors made a limited study in 1936 (unpublished) of incubation temperatures ranging from 40° to 49°C., for selective growth of *Escherichia coli* and other members of the coliform group. They used their own medium consisting of peptone, buffers, sodium chloride and glucose. Their observations led them to believe that a temperature greater than 45°C. (temperature of the medium, not of the incubator) was essential for the suppression of coliform types other than *Escherichia coli*.

One of the authors (A. A. Hajna, 1937) investigated the ability of bacteria of the genus *Escherichia* to decompose various carbohydrates other than glucose at 46°C. The substances tested were (monosaccharides) mannose, fructose, arabinose, xylose; (disaccharides) maltose, lactose, sucrose, trehalose; and (alcohols) mannitol, dulcitol and adonitol. It was found that with the exception of dulcitol and adonitol, all three substances were fermented at 46°C. as readily as at 37°C. They were substituted for glucose in the basic medium of Perry and Hajna mentioned above.

Hajna also observed that buffers enhanced gas production by the *Escherichia* organisms both at 37°C. and at 46°C. whereas meat extract restrained it. *Aerobacter* and *Citrobacter* cultures, however, were not tried.

PURPOSE OF PRESENT STUDY

The primary purpose of the present investigation has been to determine if incubation at temperatures less than 46°C. could be used as a selective test for *Escherichia coli*. A second objective was the determination of the comparative value of MacConkey's broth (as suggested by Wilson and Dodgson) and the authors' modified Eijkman lactose broth at 44°C. Since glucose, lactose and mannitol have all been found valuable and have been recom-

mended at times, these three carbohydrates were used. Temperatures of 42°, 44° and 46°C. were considered to constitute an adequate range for the purpose of this study.

CULTURES AND METHODS

Nearly all cultures[1] of the coliform group were freshly isolated from various materials under routine investigation. The *Aerobacter* and *Citrobacter* strains were alternately subjected to serial transfers in Koser's synthetic citrate medium and on Levine's eosin methylene-blue agar plates until their purity was beyond question. All of the cultures were grouped according to their biochemical reactions in various differential media (see table I).

Tests for indol were made with Ehrlich's reagent, on 18 to 24-hour cultures grown in Bacto-tryptone broth.

Aerobacter aerogenes was differentiated from *Aerobacter cloacae* by means of the hippurate test rather than by the time-consuming gelatin liquefaction test. Hippurate was considered to be hydrolyzed if cultures grew and caused definite turbidity in the synthetic medium devised for this test by Hajna and Damon (1934). If the medium remained clear at the end of three days incubation at 37°C., the reaction was considered negative.

The composition of the authors' modified Eijkman medium is:

Peptone (Bacto)	15 grams
Sodium chloride	5 grams
Dipotassium hydrogen phosphate (anhydrous)	4 grams
Potassium dihydrogen phosphate (anhydrous)	1.5 grams
Lactose, glucose or mannitol	3 grams
Distilled water	1 liter

The final hydrogen-ion concentration of the medium without titration is approximately 6.9.

The formula of the MacConkey broth as used by Wilson is:

Peptone (Bacto)	20 grams
Sodium chloride	5 grams
Lactose	10 grams
Sodium taurocholate	5 grams
Distilled water	1 liter

[1] The authors wish to thank Dr. Ralph Tittsler of the University of Rochester, for some of the *Aerobacter* cultures and for 24 *Citrobacter* cultures.

Each liter of broth was titrated to pH 7.4 and filtered before 10 ml. of 1 per cent aqueous solution of neutral red was added.

Both media were tubed in approximately 5 ml. amounts, with inner fermentation tubes. The inoculated tubes of media were placed in wire test-tube racks throughout the experiments.

GAS PRODUCTION FROM GLUCOSE, LACTOSE AND MANNITOL BY COLIFORM BACTERIA AT TEMPERATURES OF 42°, 44° AND 46°C.

All of the inoculations were made by a loop from 24-hour Bacto-tryptone broth cultures. Gas production was noted at 24 and 48 hours. The final readings as given in the tables are those made at 48 hours. Only one temperature (46°C.) was used for the *Escherichia* organisms since at that temperature, they fermented the carbohydrates and alcohols with ease and it was pointless to use lower temperatures. Incubation was in a Castle air incubator (the temperature being that of the medium, not of the incubator).

Most of the *Aerobacter aerogenes* strains produced gas from all three substances, glucose, lactose and mannitol at 42°C.; many at 44°C., but few at 46°C. The same temperature selection held for both the *Aerobacter cloacae* and the *Citrobacter* strains except that none of them produced gas from either glucose or lactose at 46°C.

On the other hand, all of the 1,374 *Escherichia* organisms produced gas from mannitol at 46°C.; only four failed in glucose and five in lactose, though growth was heavy. The strains failing at 46°C. were all isolated from raw sewage.

COMPARISON OF MACCONKEY'S MEDIUM AND THE AUTHORS' MODIFIED EIJKMAN LACTOSE MEDIUM AT 44°C.

In the comparative study of MacConkey's broth with that of the authors, two tubes of each medium were inoculated from the same broth culture. One set of the tubes for each medium was incubated in the Castle air incubator while the other set was incubated in a water-bath. A United States Bureau of Standards thermometer was used to register correct temperatures. A

TABLE 1

Gas production at 42°, 44° and 46°C. by coliform bacteria

COLIFORM GROUP	SOURCES OF STRAINS	BIOCHEMICAL REACTIONS IN DIFFERENTIAL MEDIA								NUMBER OF STRAINS	TUBE TEMPERATURES OF MEDIUM								
											42°C.			44°C.			46°C.		
		MR	VP	I	Gly.	Gel.	Cit.	Uric	Hipp.		D	L	M	D	L	M	D	L	M
Aerogenes	Human feces 29, sewage 24, water 23, crabmeat 10, oysters 24, grain 5, soil 5.	−	+	−	+	−	+	+	+	120	106	103	107	77	88	102	3	5	13
Cloacae	Human feces 23, sewage 15, drinking water 12, crabmeat 2, oysters 8.	−	+	−	−	+	+	+	−	60	14	26	39	5	4	10	0	0	1
Citrobacter	Various	+	−	−42 +3	+42 −3	−	+	−	−	45	39	16	37	22	5	29	0	0	1
Escherichia	Human feces 395, animal feces 134, crabmeat 16, oysters 68, oyster waters 112, drinking waters 196, swimming pool waters 23, raw sewage 430.	+	−	+1,344 −30	−	−	−	−	−	1,374							1,370	1,369	1,374

Legend: D = dextrose, L = lactose, M = mannitol, MR = methyl red, VP = Voges Proskauer, I = indol, Gly. = glycerol, Gel. = gelatin, Cit. = citrate, Uric = uric acid test, Hipp. = hippurate.

fluctuation of 0.5°C. above and below 44°C. was noted in the water-bath, whereas in the air incubator the fluctuation in the tube temperature was 0.3°C.

Wilson contended that the temperature in a water-jacketed incubator is never quite uniform. He advocated the use of a water-bath in which the tubes are practically immersed, the water being set at 44°C. The authors' experience indicates that an air incubator is preferable to a water-bath. The lag of an hour or two before the tubes reach the high temperature, is actually desirable since it apparently permits the *Escherichia* organisms to acclimatize themselves to the higher temperature.

TABLE 2

Gas production from lactose at 44°C. in two types of media by bacteria of the coliform group

COLIFORM GROUP	NUMBER OF STRAINS INOCULATED	TYPE OF INCUBATION			
		Air (water-jacketed)		Water-bath	
		Eijkman	MacConkey	Eijkman	MacConkey
Aerogenes	122	85	55	69	33
Cloacae	62	3	3	5	3
Citrobacter	45	1	1	0	0
Escherichia	66*	66	64	66	50

* Of human origin.

All of the *Escherichia* strains (66 of human origin) produced gas from lactose in the authors' medium at 44°C. whether the tests were incubated in an air incubator or in a water bath, while in MacConkey's medium, 2 failed to produce gas from the incubator tubes and 16 from the water-bath tubes. Most of the *Aerobacter cloacae* (62) and *Citrobacter* (45) strains failed to produce gas at this temperature in both the water-bath and the air incubator. However, many of the *Aerobacter aerogenes* strains grew and produced gas at this temperature. Many more of the *Aerobacter aerogenes* organisms, however, were inhibited in MacConkey's than in the authors' medium.

Most of the tubes of MacConkey's medium had an average of about 10 to 20 per cent of gas in the inner tubes, in contrast to

the average of 30 to 60 per cent in the authors' medium. Very few of the cultures of the entire coliform group could be successfully re-isolated from MacConkey's medium at the end of 48 hours of incubation at 44°C. *Escherichia coli*, however, is readily recovered from the authors' medium after 96 hours and longer.

DISCUSSION AND CONCLUSION

Shellfish, water, sewage and other materials usually harbor a mixture of various coliform bacteria. In order to isolate *Escherichia coli* from these substances, it is necessary to suppress the growth of other coliform types. The only method which has so far been found practical for the isolation of *Escherichia coli* from materials harboring other coliform organisms is incubation at a temperature high enough to suppress the growth of such interfering coliform types. At a temperature of 45° to 46°C., coliform types other than *Escherichia coli* usually fail to grow and produce gas from a suitable carbohydrate. *Escherichia coli* strains, on the other hand, seldom fail to grow and produce gas if a suitable medium is properly used. The isolation of *Escherichia coli* from such gas tubes by streaking on to plates of eosin methylene-blue agar medium is relatively simple. Almost pure cultures are usually obtained on such plates, whereas plates made from gas tubes obtained at temperatures of 44°C. or at 37°C. frequently contain a mixture of spreading mucoid colonies of *Aerobacter aerogenes* or *Aerobacter cloacae* which are apt to have overgrown any *Escherichia coli* present. Further difficulty may arise from inability to distinguish *Citrobacter* colonies from those of *Escherichia coli*. Temperatures between 45° and 46°C. inhibit the *Citrobacter* types and eliminate this difficulty.

The authors (1933) studied the unsatisfactory features of the Eijkman test. The original Eijkman medium contained a large amount of glucose which resulted in the rapid formation of a lethal amount of acid. They recommended the reduction of the carbohydrate to 0.3 per cent and the addition of phosphates to neutralize the acid formed from the carbohydrate. The modified Eijkman lactose broth has been found superior to MacConkey's. The superiority appears to be due to the lower

concentration of carbohydrate, the presence of suitable buffers, and to the use of a water-jacketed air incubator.

An air incubator, if properly controlled, has been found preferable to incubation in a water-bath since more strains of *Escherichia coli* grow and produce gas. A number of strains of *Escherichia coli* in our series, failed to produce gas from MacConkey's medium in a water-bath at 44°C., while many of the *Aerobacter aerogenes* types grew at this temperature. Most, but not all, of the *Escherichia coli* strains grew and produced gas at 44°C. in MacConkey's medium, in an air incubator, but more of the *Aerobacter aerogenes* strains also grew in the air incubator than in the water-bath.

Our findings are, therefore, not in complete agreement with Wilson's who claimed MacConkey's medium to be highly selective for *Escherichia coli* under these conditions. It should be borne in mind, however, that organisms of the *Aerobacter* group are not usually found, to a large extent, in shellfish except under certain conditions. For this reason, temperatures as low as 44°C. may be found practical even though not completely selective. Since in our experience, an incubator temperature between 45.5° and 46°C. (temperature of medium in tubes will be between 45.2 to 45.7°C.) is easily obtained with a good water-jacketed incubator, and since *Escherichia coli* is not suppressed to any appreciable extent in the authors' medium whereas practically all strains of *Citrobacter*, *Aerobacter cloacae* and *Aerobacter aerogenes* are suppressed, we believe this temperature is most satisfactory.

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SOME GROWTH FACTORS FOR HEMOLYTIC STREPTOCOCCI[1]

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For some time we have been interested in culturing hemolytic streptococci on a medium of known composition. Such a medium has not yet been developed for any of these organisms. The nearest approach to this goal has been reported by Rane and Subbarow (1938) for the Dochez NY5 strain of hemolytic streptococci. The earlier attempts to discover the necessary growth factors have been summarized by Hutner (1938).

Our method of attack on the problem has been to treat a complex medium in some way to selectively destroy certain factors, and then to attempt the identification of these necessary compounds. Alkali treatment of a complete medium was found to destroy its ability to support growth. However, additions of riboflavin, pantothenic acid concentrates, and one of a number of reducing substances restored the medium to its former effectiveness. The need for riboflavin exhibited by various hemolytic streptococci, but not by all of the organisms used in this study, has been previously reported (Knight, 1938; Rane and Subbarow, 1938; Orla-Jensen, Otte, and Kjaer, 1936). The requirement for pantothenic acid and for a reducing compound has not been observed before.

EXPERIMENTAL PART

Cultures and assay methods

The organism used mostly in this study was *Streptococcus epidemicus* strain X40. Later in the study several other strains

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

of *S. epidemicus*, as well as other species of hemolytic streptococci, were used in order to test the applicability of our findings on strain X40 to related organisms. Unless otherwise specified, all observations were made on the X40 strain.

For assay purposes, tubes containing 20 ml. of the medium to be tested were inoculated with a small standard loopful of a 12-hour culture of the organisms in the Bactotryptone-liver extract-glucose-salts medium described below. The tubes were maintained at 37° for 36 hours, and were then examined quantitatively for turbidity in an Evelyn photoelectric colorimeter (Evelyn, 1936). Tubes of uninoculated media of the same composition as those being assayed were used to set the instrument at a reading of 100. The media in which growth had occurred were turbid and hence gave readings of less than 100, and the reading obtained served as a quantitative measure of the amount of growth. Tests showed that maximum turbidity was obtained after about 24 hours, and that the reading then remained constant for several days.

TABLE 1

Stimulatory effect of riboflavin, pantothenic acid, and reduced iron

	COLORIMETER READING
(1) Untreated medium	75
(2) Alkali-treated medium	99
(3) (2) + pantothenic acid + reduced iron	99
(4) (2) + pantothenic acid + riboflavin	94
(5) (2) + riboflavin + reduced iron	97
(6) (4) + reduced iron . . .	65

Riboflavin and pantothenic acid each one microgram per ml.; reduced iron 200 micrograms per ml.

Media

The organism grew fairly well (see table 1) in a medium containing Bactotryptone 0.5 per cent, liver extract[2] 0.02 per cent, NaCl 0.5 per cent, $MgSO_4 \cdot 7H_2O$ 0.04 per cent, $CaCl_2$ 0.01 per

[2] We wish to thank Dr. David Klein of the Wilson Laboratories for generous gifts of liver extract.

cent, K_2HPO_4 0.1 per cent, glucose 0.1 per cent. A basal medium, on which no growth occurred, was produced by adjusting the above medium (minus glucose and phosphate) to pH 13, autoclaving for 15 minutes, and acidifying. The acid solution was concentrated under reduced pressure and made up to the original volume. Glucose and phosphate were then added and the pH was adjusted to 7.4. The concentration of the acid solution was carried out to remove H_2S formed from alkali-labile —SH compounds. This was necessary in order to demonstrate the effect of sulfide (see below).

Effect of lactoflavin, pantothenic acid, and reduced iron

Preliminary experiments with adsorbents had indicated the need for lactoflavin. For example, if the liver extract were adsorbed on norit, and the adsorbate eluted with pyridine and alcohol, the eluate plus filtrate would not support growth, but these two plus riboflavin gave good growth.

The alkali-labile growth factors that have been recognized are lactoflavin, pantothenic acid, thiamin and cozymase. Additions of these compounds to the basal medium did not make growth possible. However, when reduced iron (finely-divided elemental iron) was added, good growth was obtained when lactoflavin and pantothenic acid were the only other additions. Cozymase[3] and thiamin had no detectable effect. The data showing the effects of all three accessory factors, and of each possible pair, are given in table 1.

Is pantothenic acid the active material in the concentrates?

Since pantothenic acid has not been obtained pure, the question arises as to whether the activity of the pantothenic acid concentrates is due to that substance, or to some other material. Fortunately, through the courtesy of Dr. R. J. Williams and Dr. E. E. Snell, we were able to obtain pantothenic acid concentrates of varying degrees of potency which had been made by two different procedures (Williams *et al.*, 1938; Snell *et al.*, 1938). Calcium

[3] The cozymase was a pure preparation of von Euler, kindly supplied by A. E. Axelrod.

pantothenate of 40 and 80 per cent purity was obtained from Dr. Williams, and material found to be 1 per cent pantothenic acid (assayed by Dr. Snell) was prepared according to the method of Snell, Strong, and Peterson (1937). From the data in table 2, it can be seen that the activities of these preparations were proportional to their pantothenic acid contents.

Williams (1938) has shown that pantothenic acid is composed of β-alanine united to an hydroxy acid in amide linkage. With this formula in mind, we attempted to inactivate our concentrate with alkali, extract out the acid fragment, and recombine it with

TABLE 2

Comparison of the effect of various pantothenic acid concentrates

	AMOUNT OF CONCENTRATE ADDED	AMOUNT OF PANTOTHENIC ACID ADDED	COLORIMETER READING
	micrograms per ml.	*micrograms per ml.*	
40 per cent calcium pantothenate	0.025	0.01	80
	0.25	0.1	65
80 per cent calcium pantothenate	0.012	0.01	77
	0.12	0.1	62
1 per cent pantothenic acid	1	0.01	78
	10	0.1	65
Resynthesized liver concentrate	10		77
Synthesis from deaminated lysine	10		80

β-alanine to yield an active material. This was done as follows. The pantothenic acid concentrate prepared by the method of Snell, Strong, and Peterson was heated on the steam bath in normal alkali for 1 hour. The solution was then acidified and extracted with ethyl acetate. The extract was acetylated (to protect the hydroxyl groups) and then treated with thionyl chloride. The resulting mixture of acid chlorides was added to a pyridine solution of β-alanine ethyl ester. The pyridine was next removed under reduced pressure, the residue taken up in dilute HCl, and the solution extracted with ethyl acetate. The extracted material was allowed to stand in alcoholic alkali for 1

hour at room temperature in order to remove the ester and acetyl groups. (The ester-acetates were inactive.) After neutralization, the material was assayed and found to be active.

A purely synthetic preparation of what may be a homolog of pantothenic acid was made as follows. Lysine was deaminated, and the hydroxy acid extracted with ethyl acetate. When this acid was carried through a similar procedure to the above, a product was obtained which had about 0.1 per cent of the activity of pantothenic acid. The ester-acetate of this material was inactive.

TABLE 3

Response to various amounts of riboflavin and pantothenic acid

PANTOTHENIC ACID	RIBOFLAVIN	REDUCED IRON	COLORIMETER READING
micrograms per ml.	*micrograms per ml.*	*micrograms per ml.*	
0.001	1	200	86
0.01	1	200	75
0.1	1	200	68
0.5	1	200	66
1.0	1	200	65
1.0	0.001	200	80
1.0	0.01	200	75
1.0	0.1	200	68
1.0	1	200	70

These experiments strongly indicate that pantothenic acid is the active substance in the concentrates. Furthermore, they seem to indicate that the active substance is composed of an hydroxy acid and β-alanine, and thus conform to Williams' views on the structure of pantothenic acid.

Optimum concentrations of riboflavin and pantothenic acid

In order to determine the response to various levels of riboflavin and pantothenic acid, the basal medium was supplemented with 1 microgram per ml. of one, and the concentration of the other was varied. By this technique, it appeared that 0.1 microgram per ml. of riboflavin and 1 microgram per ml. of pantothenic acid was adequate. In some runs 0.1 microgram per

ml. of pantothenic acid gave just as good results as the 1 microgram level. Representative data are tabulated in table 3.

The need for reducing substances

It was noted above that the basal medium plus riboflavin and pantothenic acid supported good growth only when reduced iron was added. Sodium sulfide itself gave no stimulation, but when added along with the iron, more growth was obtained than when the iron alone was used. Various other reducing substances could replace the iron, although they did not seem to be quite as effective. Thus, vitamin C, glutathione, or thioglycollic acid produced some effect (table 4). In order to avoid destruction

TABLE 4

Effect of various reducing substances

COMPOUND	AMOUNT ADDED	COLORIMETER READING
	micrograms per ml.	
Reduced iron	200	65
NaSH	100	98
Reduced iron + NaSH	200 + 100	60
Vitamin C	100	81
Glutathione	100	79
Thioglycollic acid	100	82

Each tube contained 0.1 microgram each of pantothenic acid and riboflavin in addition to the alkali-treated medium.

by heat, these compounds were sterilized by filtration and added aseptically to the tubes just before inoculation.

The results illustrated in table 4 are in harmony with the hypothesis that alkali-labile reducing substanees were present in the liver extract or tryptone. In view of the unspecific nature of the reducing substance, it would be interesting to try the effect of reduced iron on organisms reported to require vitamin C, or glutathione.

Application to other hemolytic streptococci

In order to test the general applicability of the results obtained with *S. epidemicus* strain X40, a number of other hemolytic

streptococci were used. Three additional strains of *S. epidemicus*, C108, X32, and W116–7 were found to have requirements similar to those of X40. The effect of sodium sulfide was most clearly shown in the case of W116–7 (table 5).

Streptococcus pyogenes strain J17A4 (Lancefield type A) and *Streptococcus equi* strain F132 (Lancefield type C) behaved as did the strains of *S. epidemicus,* except that with *S. pyogenes* the need for sodium sulfide was pronounced. A Lancefield type B organism, *Streptococcus mastitidis* strain O-90R, and a Lancefield type D, *Streptococcus zymogenes* strain H-6905, failed to grow on the alkali-treated medium, but grew quite well if any two of the

TABLE 5

Comparison of the response obtained with various streptococci

MEDIA	COLORIMETER READINGS OBTAINED WITH				
	S. epidemicus			*S. pyogenes*	*S. zymogenes*
	X32	C108	W116-7		
(1) Alkali-treated basal	100	100	100	100	96
(2) (1) + riboflavin + pantothenic acid	93	93	96	100	65
(3) (1) + riboflavin + reduced iron	100	97	100	100	72
(4) (1) + pantothenic acid + reduced iron	95	96	100	100	90
(5) (2) + reduced iron	70	58	95	100	45
(6) (5) + sodium sulfide	66	58	73	55	56

Riboflavin and pantothenic acid each 0.1 microgram per ml.; reduced iron and sodium sulfide each 100 micrograms per ml.

factors were added. However, better growth was obtained by supplying all three compounds. A Lancefield type F (strain H60R) refused to grow even on the untreated basal medium. Representative data are shown in table 5.

SUMMARY

By the use of an alikali-treated medium it has been shown that a number of hemolytic streptococci require riboflavin, pantothenic acid, and a suitable reducing compound. No reducing substance was found to be specific, for vitamin C, glutathione, thioglycollic acid, or reduced iron were each found to be effective for *Strepto-*

coccus epidemicus. For most of the organisms examined, sodium sulfide was beneficial, and for a few it appeared to be essential. A few organisms were found which required some, but not all, of the alkali-labile factors.

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GROWTH FACTORS FOR BACTERIA

VIII. Pantothenic and Nicotinic Acids as Essential Growth Factors for Lactic and Propionic Acid Bacteria[1]

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The preparation of highly active concentrates of a substance essential for the growth of all lactic acid bacteria tested was described by Snell, Strong and Peterson (1937). Further attempts to purify this substance are reported below, together with results which show that the active substance is pantothenic acid (Williams (1938a), cf. Snell *et al.* (1938a)). Nicotinic acid is also shown to be essential for some species of lactic acid bacteria.

EXPERIMENTAL

Methods

The basal media A and B, the cultures used, and the details of carrying out the fermentations have been previously described (Snell *et al.* (1937)). Medium A must be supplemented with tryptophane, and B with riboflavin, for use with organisms which require these substances (Snell and Strong (1938b)).

Bacterial response has been determined in the present work by measuring acid production or in some cases by measuring relative turbidity by the photoelectric colorimeter (Evelyn (1936)). Light transmission of the uninoculated medium was adjusted to 100, and the per cent of this transmitted by the inoculated tubes after 24 to 48 hours incubation was read directly from the gal-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

vanometer scale. Overhead lights must be off while such readings are being made to prevent reflection of stray light into the photoelectric cell by the turbid suspension. The 540 mμ filter was used with medium B because at this wave-length light absorption of the medium was only slightly greater than that of distilled water, and turbidities could thus be measured with less interference.

Identity of pantothenic acid with the growth factor for lactic acid bacteria

Through the kindness of Professor R. J. Williams it was possible to compare two preparations of pantothenic acid with our liver concentrates. These samples, designated I and II, contained 40 and 83 per cent respectively of calcium pantothenate. Sample I replaced the standard liver extract (Snell *et al.* (1937)), and contained one unit[2] of activity in approximately 0.15 microgram (table 1). The activity of sample I, like that of our liver concentrate, was destroyed by heating in 0.05N NaOH at 100° for one hour.

The effects of I and II in promoting growth and acid production by *Lactobacillus casei* are compared in table 2. The activity of II was almost exactly twice that of I. The latter was easily detectable at 0.001 microgram per ml. One unit of pure pantothenic acid would correspond to approximately 0.06 microgram.

The action of I and II on nine other species of lactic acid bacteria, all of which require our growth factor, is shown in table 3. Sample II completely replaced the liver fractions except for *Lactobacillus delbrückii* and *Lactobacillus mannitopeous*. Some activity was evident in these cases, but growth did not take place on subculture into the same medium, even though a large excess of I (100 micrograms per 10 ml.) was added. Evidently some other growth factor is required by these two species. All of the other species could be subcultured repeatedly in the medium

[2] One unit of activity is defined as that weight of material which must be added to 10 ml. of medium to produce a response equal to that given by 0.05 mgm. of a standard liver preparation. With *L. casei*, 1 unit of the growth factor gives approximately ½ maximum fermentation under the conditions used.

supplemented with I; no growth occurred in its absence. The behaviour of *L. delbrückii* lessened its utility for assays, and *L.*

TABLE 1

*Comparison of activity of 40 per cent calcium pantothenate (sample I) with standard liver preparation**

STANDARD LIVER PREPARATION		40 PER CENT CALCIUM PANTOTHENATE (SAMPLE I)			
		Untreated		Alkali Treated	
Amount added per 10 ml.	Incident light transmitted	Amount added per 10 ml.	Incident light transmitted	Amount added per 10 ml.	Incident light transmitted
micrograms	*per cent*	*micrograms*	*per cent*	*micrograms*	*per cent*
0	93	0	91	0	93
30†	71	0.05	86	1.0	93
60	62	0.10	79	3.0	92
150	46	0.30	52	5.0	93
		0.50	44	10.0	94
		1.0	40		
		5.0	41		

* *Lactobacillus casei* in Medium B. Incubation time, 30 hours at 37°C.
† One unit.

TABLE 2

*Quantitative comparison of the activity of samples I and II in promoting growth and acid production of Lactobacillus casei**

40 PER CENT CALCIUM PANTOTHENATE (I)			83 PER CENT CALCIUM PANTOTHENATE (II)		
Amount added per 10 ml.	Acid produced per 10 ml.	Visible growth†	Amount added per 10 ml.	Acid produced per 10 ml.	Visible growth
micrograms	*cc. 0.1 N*		*micrograms*	*cc. 0.1 N*	
0.00	0.5	−	0.00	0.5	−
0.05	1.2	+	0.025	1.6	+
0.10	3.5	++	0.05	3.3	++
0.30	5.6	+++	0.15	5.8	+++
0.50	6.8	++++	0.25	7.2	++++

* Medium B. Incubation time, 4 days at 37°C.
† − indicates no visible growth; + slight growth; ++ fair growth; +++ heavy growth; ++++ very heavy growth (with sediment).

casei was substituted for it in later work. Table 3 again shows sample II to be approximately twice as active as sample I.

The similarity between chemical properties of our growth factor

TABLE 3

*Effect of pantothenic acid concentrates on other species of lactic acid bacteria**

ORGANISM	SUPPLEMENT TO MEDIUM					ACTIVITY							
	Sample	Micrograms per 10 ml.				cc. 0.1 N acid per 10 ml. medium				Visible growth†			
		a	b	c	d	a	b	c	d	a	b	c	d
Lactobacillus arabinosus	I	0	0.3	0.5	1.0	0.8	7.7	8.4	8.4	−	++++	++++	++++
	II	0.05	0.1	0.3	0.5	5.2	6.3	8.5	8.4	+++	++++	++++	++++
Lactobacillus delbrückii	II	0	0.1	0.3	0.5	1.2	2.4	3.4	2.5	+	++	++	++
Lactobacillus mannitopoeus	II	0	0.1	0.3	0.5	0.3	0.5	0.9	1.2	−	−	+	+
Lactobacillus pentosus	II	0	0.1	0.3	0.5	1.2	4.2	7.6	8.3	+	+++	++++	++++
Bacillus brassicae	I	0	0.3	0.5	1.0	0.6	7.4	8.5	8.4	−	++++	++++	++++
	II	0.05	0.1	0.3	0.5	5.9	6.7	8.4	8.4	+++	++++	++++	++++
Bacillus lactis-acidi	I	0	0.3	0.5	1.0	0.8	3.6	4.9	6.8	−	+++	+++	++++
	II	0.05	0.1	0.3	0.5	2.1	3.3	4.8	5.3	++	+++	+++	++++
Leuconostoc mesenteroides	II	0	0.1	0.3	0.5	0.9	2.0	3.4	4.3	+	+++	++++	++++
Streptococcus lactis	II	0	0.1	0.3	0.5	0.3	1.5	2.5	2.7	−	++	+++	+++
Propionibacterium pentosaceum	II	0	0.1	0.3	0.5	0.3	0.9	1.9	2.2	−	++	++++	++++

* Medium A.

Incubation time, 4 days at 37 or 28°C.

† As in table 2.

and those of crude pantothenic acid has been borne out by recent reports of Williams and co-workers (1938a, b). Both substances are inactivated by alkali or acid, by acetylation or esterification, and both are comparatively resistant to oxidation by hydrogen peroxide. Further evidence for the identity of the two consists in the partial synthesis of pantothenic acid described below. The active substance in the liver preparations thus appears to be identical with pantothenic acid, and will be so designated.

TABLE 4

Stability of pantothenic acid in the alkaline range

pH	TIME OF TREATMENT*		ACTIVITY
	At room temperature	At 100°C.	
	hours	*hours*	*micrograms per unit*
7.0	0	0	1
7.0	22	2	1
8.0	22	2	1
9.0	22	2	1
10.0	5	0	1
	24	0	1
	4	1	1.3
	22	2	1
11.0	5	0	1
	24	0	1
	4	1	3.5
	22	2	5.7

* Samples were held first at room temperature, then at 100° for the time indicated.

A modified procedure for preparing pantothenic acid concentrates

Assays for pantothenic acid have been made on medium B with *L. casei* because this organism grows well on repeated subculture in medium B plus pantothenic acid, and thus obviates complications arising from lack of unrecognized nutritive factors.

To determine more accurately the alkali stability of the active substance, a liver preparation containing one unit of activity per microgram (approximately six per cent pantothenic acid) was treated with various buffers as indicated in table 4. The activity was retained at pH 11 at room temperature for 24 hours, but was diminished at pH 10 when the mixture was heated to 100°.

Almost none of the active substance was adsorbed from liver extracts by an equal weight of norite at pH 9, although the solution was almost entirely decolorized. At pH 3 or 4 adsorption was almost quantitative. On the basis of these results a modified procedure, avoiding use of lead acetate, was developed.

One hundred fifty grams of the alcohol-soluble liver fraction[3] (1 unit in 0.05 mgm.) was dissolved in 500 ml. of water, filtered, and diluted to seven liters. NaOH was added to pH 9, 120 grams of Pfansteihl Norit A added, the mixture stirred for one hour, and filtered. The filtrate was adjusted to pH 3.8 with H_2SO_4, 95 grams of norite added and the mixture again stirred for one hour. The norite was filtered off, washed once in water, then eluted three times by stirring for 15 minutes with 500-ml. portions of a pyridine-ethanol-water mixture (1:1:2). The solvents were removed below 40° by distillation under reduced pressure. The eluate (about 18 to 20 grams) was dissolved in 100 ml. of water, adjusted to pH 6.3 with NaOH, and extracted continuously with ether for two days. The inactive extract was discarded, the pH of the residue adjusted to 1.0 with H_2SO_4, and ether extraction continued for 48 hours. The extract, approximately 3.3 grams., contained one unit of activity in 0.8 microgram (approximately 6.5 per cent pantothenic acid), which accounts for about 90 per cent of the original activity. Attempts to carry out this procedure on kilogram batches of material resulted in much poorer yields, probably because of destruction during ether extraction.

The ether extract may be further purified by reëxtraction with ether, or by salt fractionation.

Fractionation of the sodium salts of the ether extract

To 1.3 grams of an ether extract in water solution, NaOH was added to pH 7.5. The solution was concentrated to dryness, and the sodium salts were extracted with two 25 ml. portions of absolute alcohol, which removed 1.02 grams. To 200 mgm. of this preparation dissolved in 15 ml. of absolute alcohol, was added

[3] That portion of an aqueous extract of liver soluble in 92 per cent alcohol. The authors wish to thank Dr. David Klein of Wilson and Company, Chicago, for supplying this material.

15 ml. of acetone, and the resulting precipitate (A) centrifuged out. The supernatant liquid was concentrated to dryness, the residue dissolved in 7 ml. of absolute alcohol, and 18 ml. of acetone added. The precipitate (B) was removed, the supernatant liquid concentrated to dryness, the residue dissolved in 5 ml. absolute alcohol, and 50 ml. of acetone added. The precipitate (C) was centrifuged out, and the mother liquors concentrated to dryness (D).

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	200	0.83	241,000	
A	32.6	3.0	10,900	4.5
B	40.0	0.9	44,400	18.4
C	45.0	0.4	112,500	46.7
D	67.0	1.5	44,700	18.6

Eighty-eight per cent of the original activity was recovered in the four fractions. The most active fraction was not precipitated from absolute alcohol by 72 per cent acetone, but was precipitated by 91 per cent acetone.

Fractionation of the barium salts of the ether extract

To a solution of 2.65 grams of ether extract (1 unit in 2.0 micrograms; 3.1 per cent pantothenic acid) $Ba(OH)_2$ was added to pH 7.5, the solution concentrated to 25 ml., and 300 ml. of alcohol added slowly with stirring. The insoluble material was centrifuged out, washed with alcohol, and discarded. The supernatant liquid and washings were concentrated to dryness, the residue was dissolved in 10 ml. of water and barium removed exactly by adding H_2SO_4 and centrifuging. The solution of free acids was concentrated to dryness under reduced pressure and gave 259.2 mgm. of a light yellow syrup, which contained one unit of activity in 0.25 microgram (25 per cent pantothenic acid). An almost 10-fold concentration and 78 per cent recovery of activity was obtained. This procedure regularly gave concentrates containing 10 to 15 per cent of pantothenic acid, and often

as above, better. This fraction represents the purest preparation of pantothenic acid obtained in this work.

Organic derivatives of pantothenic acid

(a) *Acetyl derivatives.* To 78.8 mgm. of ether extract was added 0.5 ml. acetic anhydride and 1 ml. of dry pyridine. The mixture was heated one hour at 100°, and evaporated to dryness under reduced pressure. The residue weighed 99.2 mgm. and was completely inactive. The acetyl derivative was hydrolyzed by heating in a sealed tube at 100° for one hour with a methyl alcohol solution of NH_3 (saturated at 0°). Some of the unacetylated material was treated in the same way as a control.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	77.8	1.2	65,600	
Acetylated material	99.2	Inactive		
Hydrolyzed acetylated material		1.4*	70,800	107
Hydrolyzed starting material	78.8	1.4	56,200	86

* Expressed in terms of acetylated material.

Although ether solubility was greatly increased by acetylation, no fractionation was effected by use of this property.

(b) *Methyl ester.* 110 mgm. of ether extract were dissolved in 5 ml. of methanol, cooled to 0° and an excess of diazomethane in ether added. Nitrogen evolution was slow. After one-half hour evolution of gas ceased and the solution was concentrated to dryness.

Hydrolysis with alcoholic ammonia gave inconsistent results, but the ester was successfully hydrolyzed as follows: to a solution of 9.5 mgm. in 5 ml. of methanol was added 5 ml. of 2.4 N KOH in methanol. One aliquot was allowed to stand at 25° for one hour, another was heated at 100° for 40 minutes. Each was neutralized with HCl in methanol, and assayed. Suitable controls assayed at the same time showed that the KCl or methanol in the concentrations necessary for the assay had no inhibitory effect on growth.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	110	1.7	64,700	
Esterified material	95.1	Inactive		
Esters hydrolyzed at 25°		1.7*	55,900	87
Esters hydrolyzed at 100°		Inactive		0

* Expressed in terms of esterified material.

This hydrolysis procedure was also successfully applied to the acetyl derivatives.

(*c*) *Methyl ester of the acetyl derivative.* 1.16 grams of ether extract acetylated as above were dissolved in a mixture of water and ether, and the ether layer shaken five times with equal volumes of water. Evaporation of the ether left 0.252 gram of a transparent, red-brown, oily liquid. The aqueous solution was concentrated to dryness at reduced pressure, the residue dissolved in 60 ml. of absolute methanol, and 345 mgm. of diazomethane in ether solution added. The vigorous initial effervescence slowed and stopped after 45 minutes. The solution was concentrated to a syrup, which was taken up in water and ether and shaken out three times with equal volumes of ether. The yellow ether solution, after removal of the solvent, left 0.766 gram of a red-brown liquid. Evaporation of the aqueous solution yielded 0.433 gram of red-brown glassy solid.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	grams	*micrograms*		*per cent*
Starting material	1.16	2.3	505,000	
Ether soluble acetylated material	0.252	9.5*	26,500	5
Water soluble acetylated material	1.27	Not assayed		
Ether soluble esterified material	0.766	2.4*	319,000	63
Water soluble esterified material	0.433	3.6*	120,000	24

* Hydrolyzed with CH_3OH—KOH in each case before assaying; weights expressed in terms of unhydrolyzed material.

Distillation of pantothenic acid derivatives

Preliminary determinations of the heat stability of pantothenic acid, its methyl ester and its acetyl derivative showed that stabil-

ity to heat was markedly increased by esterification, especially acetylation. The samples were placed in test tubes, heated in an oil bath, and hydrolyzed where necessary before being assayed.

MATERIAL	HEAT TREATMENT		ONE UNIT	DESTRUCTION
	Time	Temperature		
	min.	*°C.*	*micrograms*	*per cent*
Ether extract			1.7	
Ether extract	30	111–117	2.3	29.5
Ether extract	30	145–155	29.0	94.2
Acetylated material			1.4	
Acetylated material	30	145–155	1.4	0.0
Esterified material			1.7	
Esterified material	30	111–117	2.3	29.5
Esterified material	30	145–155	3.2	48.5

In an attempt to distill the free acid 129 mgm. of an ether extract was placed in a molecular still[4] and held at 72 to 75° and 10^{-3} mm. for 2.5 hours. The colorless, oily distillate (I) was removed from the condenser and the residue held at 85° and 2×10^{-5} mm. for 1.25 hours. The distillate (II), and final residue (III) were collected separately and assayed.

	WEIGHT	ONE UNITS	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	129	0.5	358,000	
Distillate (I)	36	10.0	3,600	1.0
Distillate (II)	10	5.0	2,000	0.6
Residue (III)	71	0.45	165,000	46.1

The large loss of activity in the distillation of the free acid and the increased heat stability of the methyl ester and acetyl derivative suggested use of such derivatives for distillation. Since the methyl ester of the acetyl derivative should be most stable and volatile, it was selected for further trials.

Although distillation of some acetylated esterified material

[4] In this still the distance between the condensing surface, which was kept cold with dry ice and acetone, and the material to be distilled was approximately 0.4 cm.

in the molecular still showed that the active compound distilled without destruction, no appreciable fractionation resulted; a still with greater fractionating powers was necessary. A second sample was therefore distilled from an apparatus similar to that used by Almquist (1937). It consisted of a glass tube sealed at one end and divided by constrictions into equal compartments, A, B, and C, A being at the closed end. The sample was placed in A, and the tube evacuated to 0.1 mm. A was heated electrically to 100 to 130°, B to 60 to 70°, and C left at room temperature. After one hour, the tube was broken at the constrictions, the fractions in the compartments were removed and aliquots hydrolyzed for assay.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	553	2.1	264,000	
Fraction C	200	Inactive		
Fraction B	74.7	2.5	30,000	11
Fraction A (residue)	235.6	0.9	261,000	99

At the lower pressure, the undistilled material was most active, and considerable purification resulted. Subsequent distillation of Fraction A in the molecular still effected no increase in activity.

A partial synthesis of pantothenic acid

Lability of pantothenic acid to alkali and acid treatment is ascribed by Williams (1938a) to hydrolysis into β-alanine and a dihydroxy valeric acid. If this explanation is correct, reactivation of alkali-inactivated concentrates should follow recombination of the two components. This was accomplished as follows:

An active concentrate (38 mgm., 5.1 per cent pantothenic acid, one unit in 1.2 micrograms) was completely inactivated by heating at 100° with 10 ml. of N NaOH for one hour. After neutralization with HCl, the hydrolysate was concentrated to dryness and acetylated by heating for one hour at 100° with 3 ml. of pyridine and 10 ml. of acetic anhydride. After concentrating to dryness, 10 ml. of water was added, the mixture acidified with

H_2SO_4 (pH 2.0), and extracted continuously with ether for 2 hours. The extract was concentrated to dryness, and the acetylated acids redissolved in absolute ether. The acid chlorides of the mixture were formed by allowing the solution to stand (with occasional shaking) at room temperature for 3 hours with 0.2 gram PCl_5. Three cubic centimeters of anhydrous acetic acid were added to react with excess PCl_5, and the resulting solution concentrated to dryness *in vacuo.* To a solution of the resulting acid chlorides in absolute ether was added an ether solution containing 0.5 gram of β-alanine ethyl ester. The mixture was concentrated to dryness. The residue, which should contain the acetyl derivative of the ethyl ester of pantothenic acid, was dissolved in methanol and hydrolyzed with methyl-alcoholic KOH as described above. Assay showed one unit of activity in 1.8 micrograms (3.3 per cent pantothenic acid). Therefore, 65 per cent of the original activity was recovered by the resynthesis. In conjunction with data given above, this experiment furnishes conclusive evidence of the identity of the active factor in our liver concentrates with pantothenic acid.

Nicotinic acid as a growth factor for lactic acid bacteria

The identification of pantothenic acid as essential for growth of lactic acid bacteria made it desirable to test the ability of these organisms to grow on a more highly purified medium containing this substance. The basal medium consisted of acid-hydrolyzed, purified casein,[5] 0.5 per cent; tryptophane, 0.01 per cent; cystine, 0.01 per cent; mineral salts;[6] glucose, 1 per cent; sodium acetate, 0.6 per cent; riboflavin, 0.01 mgm. per 100 ml. The medium was tubed in 10 ml. lots, sterilized for 15 minutes at 15 pounds pressure and inoculated with the test organism. For inoculum, cells grown for 24 hours in medium B supplemented with pantothenic acid concentrates were centrifuged out and resuspended in an equal volume of 0.9 per cent NaCl solution. Each assay tube was

[5] Labco vitamin-free casein was hydrolyzed with 20 per cent H_2SO_4 at 120° for eight hours. H_2SO_4 was quantitatively removed with $Ba(OH)_2$.

[6] K_2HPO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; $MnSO_4 \cdot 3H_2O$, 0.01 gram; in 1000 ml. of medium.

inoculated with 0.05 ml. of this suspension. Of the organisms tested none made more than slight growth and this only occasionally. Such growth was probably due to carry-over of essential substances with the heavy inoculum, because it did not continue on subculture. Evidently essential substances, present in the hydrolyzed peptone of medium A, and the sodium-hydroxide-treated peptone of medium B, were lacking. That nicotinic acid is one such substance is evident from table 5. Nicotinic acid

TABLE 5

*Nicotinic acid as a growth factor for certain lactic acid bacteria**

ORGANISM	MICROGRAMS NICOTINIC ACID ADDED PER 10 ML.					ACTIVITY†				
	a	b	c	d	e	a	b	c	d	e
Lactobacillus arabinosus	0	0.1	0.5	1	3	+ 1.7	+++ 4.7	+++ 5.2	+++ 5.3	+++ 5.2
Lactobacillus casei	0	0.1	0.3	0.5	1	+ 2.2	++ 4.1	+++ 5.4	+++ 6.6	+++ 5.4
Leuconostoc mesenteroides	0	0.1	0.5	1	3	+ 1.0	+ 1.6	+ 1.2	+ 1.4	+ 1.3
Propionibacterium pentosaceum	0	0.1	0.5	1	3	+ 0.9	+ 1.2	+ 1.2	+ 1.1	+ 1.0
Streptococcus lactis	0	0.1	0.5	1	3	+ 1.0	+ 1.2	+ 1.2	+ 1.1	+ 1.1

* Incubation time, 5 days. *L. casei* at 37°C.; all other organisms at 28°C.

† –, + etc. as in table 2. Figures are ml. of 0.1 N acid produced per 10 ml. of medium.

greatly stimulated growth of *L. casei* and *Lactobacillus arabinosus* in the first culture, but growth failed on subculture, probably because of a lack of other essential growth factors. The other organisms, data for three of which are given in table 5, failed to show significantly increased growth in the presence of nicotinic acid. All grew well when small amounts of yeast, liver or malt extract were added to the basal medium. The nature of the additional factor or factors necessary for growth is being investigated. The effect of such extracts cannot be duplicated by

pimelic acid, β-alanine, uracil, pyruvic acid, vitamin B_1, vitamin B_6,[7] inositol, betaine, nicotinic acid or glutathione, alone or in various combinations.

DISCUSSION

Four lines of evidence point to the identity of the growth factor for lactic acid bacteria (Snell *et al.* (1937)) with pantothenic acid. (1) Purified preparations of pantothenic acid completely replace growth factor preparations for a variety of lactic acid bacteria. (2) 83 per cent calcium pantothenate is almost exactly twice as active as 40 per cent calcium pantothenate.[8] This relationship would be very improbable if an impurity were responsible for the effect. (3) The formula for pantothenic acid, proposed by Williams (1938a) explains the known properties of the growth-promoting factor for lactic acid bacteria. The proposed formula is that of a substituted amide, formed from β-alanine and a dihydroxy-valeric acid of unknown configuration. Hydrolysis of the amide linkage explains lability of the growth factor to acid and alkali. The presence of hydroxyl and carboxyl groups explains inactivation by acetylation and esterification. Marked water solubility and limited solubility in ether would also be expected in a compound of this type. (4) The active substance in our concentrates has been destroyed by alkaline hydrolysis, and an active compound resynthesized on the basis of the formula for pantothenic acid suggested by Williams.

The assay which has been developed for the present work appears to be a specific and quantitative method for determining pantothenic acid, and may prove useful in view of the growing biological importance of this substance.

The essential nature of pantothenic acid for *P. pentosaceum* is not in agreement with the reports from this laboratory by Wood *et al.* (1937, 1938), although the same strain of organism (no. 11, Hitchner (1934)) was used. The failure of pantothenic acid

[7] We wish to thank Dr. S. Lepkovsky for a sample of crystalline vitamin B_6.

[8] The figures given are those kindly furnished by Professor Williams. Wherever the pantothenic acid content of a fraction is given in this paper it has been referred to these values as standard.

preparations, alone or in combination with other known growth factors to replace ether extract of yeast, must be attributed to some unrecognized deficiency in the basal medium used.

Möller (1938) reported that vitamin B_6 is required by certain lactic acid bacteria. We have been unable to detect any clear-cut stimulating action of this vitamin on *L. casei*, but this result may be due to the lack of a suitable basal medium.

The number of known accessory factors for the lactic acid organisms is now raised to four (riboflavin, pantothenic acid, nicotinic acid, and vitamin B_6). It is also evident that others remain to be discovered.

SUMMARY

The factor previously described as necessary for growth of all lactic acid bacteria tested has been identified with pantothenic acid. Convenient methods for the preparation of highly active concentrates of this substance are described, together with experiments which give further information concerning its properties.

Nicotinic acid greatly stimulates growth and acid production by some but not all lactic acid bacteria, and is regarded as essential for certain of these organisms. Other unidentified factors are also required by these bacteria for growth on highly purified media.

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THE INFLUENCE OF NICOTINIC ACID ON GLUCOSE FERMENTATION BY MEMBERS OF THE COLON-TYPHOID GROUP OF BACTERIA[1]

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Knowledge concerning the enzymic systems of various pathogenic bacteria and their dependence on specific vitamins or accessory substances is still rather vague. The work of Davis (1917), Thjötta and Avery (1921), and Fildes (1921) has demonstrated that *Hemophilus influenzae* requires two substances for its growth: x, a heat-stable substance, probably hematin, and v, a heat-labile substance. Kligler (1919) showed, in an extended study on the growth requirements of certain pathogenic bacteria (*Corynebacterium diphtheriae*, meningococcus, etc.), that minute quantities of tissue extractives, or extracts of nasal mucous secretion are either essential for growth or have a stimulating effect. More recently Knight (1937) has shown that nicotinic acid was essential for the growth of *Staphylococcus aureus*, and

[1] Note: The experiments reported in this paper were carried out with the Schering-Kahlbaum peptone für bacteriologische Zwecke. Since the paper was submitted for publication, we changed over to Difco peptone and failed to duplicate the results. A comparative test with three peptones showed that work on the effect of nicotinic acid can only be carried out satisfactorily with synthetic media or with peptone free from nicotinic acid. The fermentation of glucose in media made with the different peptones is summarized below. The media contained 0.3 per cent peptone, 0.1 per cent glucose, salt solution as given in the body of the paper, and 10γ nicotinic acid per ml.:

	SCHER.-KAHL		RIEDEL		DIFCO	
	+N	−N	+N	−N	+N	−N
Shigella dysenteriae	+	−	+	+	+	+
Salmonella paratyphi A	+	−	+	+	+	+

Mueller (1937) has reported that *C. diphtheriae* can be cultivated on synthetic media if nicotinic acid and β alanin are added. Since nicotinic acid forms a part of the fermentation enzyme of Warburg and functions as a cozymase, we have undertaken a study of the relation of this substance, if any, to the fermentative capacities of the various species of the colon-typhoid-dysentery group of bacteria. A preliminary note dealing principally with *Shigella dysenteriae* (Shiga), has already been published (Kligler and Grosowitz, 1938). The object of this paper is to report the results of experiments with representative species of this group of fermenting bacteria.

EXPERIMENTAL

Methods

In order to exclude, in so far as possible, unknown substances and at the same time avoid expensive and complicated synthetic substrates, the experiments were conducted with a semi-synthetic medium. The basis of this medium was peptone, which provided a mixture of the various amino-acids required for growth; all the other ingredients were chemically pure substances. The basic solutions used throughout these experiments consisted of the following:

A. Salt mixture:

	per cent
NaCL	0.5
$Na_2HPO_4 \cdot 2H_2O$	0.25
KH_2PO_4	0.035
$MgCl_2$	0.03
Fe and Mn	trace

These salts were dissolved in distilled water and filtered through a Seitz filter.

B. A stock solution of peptone dissolved in distilled water and autoclaved.

C. The glucose and vitamin solutions respectively were dissolved in distilled water and sterilized by filtration through Seitz filters.

The liquid media were prepared by adding desired amounts of

solutions B and C to the stock salt solution. Solid media were prepared by adding the requisite amount of a stock solution of agar in distilled water, to give a concentration of two per cent. The agar-agar used was first thoroughly washed with distilled water, dried and then the stock solution made up in distilled water.

Fermentation in solid media

An orientation experiment was made by growing the various strains on a solid medium consisting of the stock salt solution,

TABLE 1

Growth and glucose fermentation in the experimental solid medium with and without nicotinic acid

STRAIN	GROWTH AND FERMENTATION		
	Without nicotinic acid	With nicotinic acid	Russell double sugar
Eberthella typhosa H 441	+	+	+
Salmonella paratyphi A	0	+	+G
Salmonella paratyphi B	+G	+G	+G
Escherichia coli	+G	+G	+G
Shigella dysenteriae Shiga (Parker strain)	0	+	+
Shigella dysenteriae Flexner	−	+	+
Shigella dysenteriae Y	−	+	+

Note: 0 = no growth; − = poor growth, no acid; + = growth & acid formation; G = gas.

0.3 per cent peptone, 0.2 per cent glucose, 2 per cent agar and andrade indicator. One set of tubes contained nicotinic acid, the other did not. The usual stab and slant inoculations were made as in Russell double sugar media. Ordinary nutrient double sugar tubes were inoculated for comparison. The results are summarized in table 1. It is apparent from the data that there are marked differences in the behavior of the different species of bacteria. In the medium without nicotinic acid the dysentery bacilli as a group either fail to grow or grow poorly without evidence of fermentation of the glucose. The same is true of paratyphoid A. The other species tested grew well and

were able to ferment glucose without the aid of nicotinic acid. It is of interest also to note that paratyphoid A produced acid but no gas in the synthetic medium containing nicotinic acid. These points will be dealt with in detail below.

It would seem that this relatively simple semi-synthetic medium with glucose and andrade indicator may serve to differentiate paratyphoid A from B and *S. dysenteriae* from *E. typhi.*

In order to ascertain whether the paratyphoid A and dysentery strains required vitamins or growth-stimulating substances other than nicotinic acid, these strains were grown on the stock

TABLE 2

Growth and glucose fermentation in media containing various vitamins with and without nicotinic acid

	SHIGA	FLEXNER	PARATYPHI A
Without nicotinic acid:			
Control	0	–	0
1γ/ ml. B_1	0	–	0
10γ/ml. B_2	0	–	0
10γ/ml. β alanin	0	–	0
10γ/ml. β alanin + B_2	0	–	0
200γ/ml. ascorbic acid	–	–	–
With nicotinic acid:			
10γ/ml. Nicotinic acid	+	+	+
N + B_1	+	+	+
N + β alanin	+	+	+
N + ascorbic acid	+	+	+
N + β alanin + B_2	+	+	+

medium described above, to which various substances were added. The results are summarized in table 2. None of the accessory substances added, with the exception of nicotinic acid, had any effect on the growth or fermentation of the bacteria used. As in the preceding experiment the dysentery strains grew in all media not containing nicotinic acid but did not produce acid. On the other hand, in the corresponding media containing nicotinic acid, all strains grew much more abundantly with the production of acid along the stab inoculation. It should be noted that in this series of media also the paratyphoid A strain failed to produce gas.

A clearer picture of the influence of nicotinic acid on those bacteria which failed to give evidence of fermentation in the solid glucose medium devoid of this substance, was obtained by noting the acid production and sugar utilization in liquid media. The medium was prepared in the manner described above. To avoid carrying over traces of accessory substances with the bacteria, small inocula were used (about 100 cells) of a broth culture diluted in saline. Where for comparison larger inocula (1 million cells) were used, the broth cultures were washed twice in saline and saline suspensions used for inoculation. All experiments were repeated at least once. Since there was practically no deviation in the results, only typical experiments are given.

The effect of various concentrations of peptone on the amount of sugar utilized

This experiment is illustrative. The media were the same in every other respect, except that they contained varying concentrations of peptone. The same inoculum was used. The glucose was determined by the Lehmann-Maquenne method.

The results are summarized in table 3. It will be noted that, to a limited extent, the concentration of peptone influenced the degree of sugar utilization. This is presumably due to the effect of peptone on the rate of growth, since more prolonged incubation decreased the differences. However, the data, whether after twenty-four or forty-eight hours incubation, show clearly that in the absence of nicotinic acid there is a small, though constant destruction of glucose (11 per cent) in all tubes, whereas in the presence of nicotinic acid over 90 per cent of the glucose is utilized in all tubes containing 0.2 per cent peptone or over. Longer incubation does not affect these results.

It is difficult to account for the small but consistent destruction of glucose in the absence of nicotinic acid. As already stated, the fact that even this degree of utilization is influenced by the concentration of peptone and the incubation time, would indicate that the loss is due to cell activity. It must be assumed, however, that this glycolysis differs in character from that taking place in the presence of nicotinic acid.

Experiments to determine the effect of nicotinic acid on cell activity as shown by the duration of the lag period did not always give consistent results. In general there were indications of a small decrease in the duration of the lag in media containing nicotinic acid, but the differences were not sufficiently marked to suggest that this substance was essential for stimulating cell metabolism other than as a cozymase in glucose fermentation.

TABLE 3

Influence of nicotinic acid on the utilization of glucose by S. dysenteriae (Shiga), in media containing varying concentrations of peptone

INCUBATION TIME AT 37°C.	CONCENTRATION OF PEPTONE	GLUCOSE REMAINING IN 9 ML. OF MEDIA: With nicotinic acid		Without nicotinic acid	
hours	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*
24	0.1	4.96	60.0	8.24	98.0
	0.2	1.82	22.0	7.50	90.0
	0.3	0.65	8.0	7.25	89.0
	0.4	0.56	7.0	7.18	89.0
	0.5	0.80	8.0	7.19	89.0
48	0.1	3.99	47.0	7.24	89.0
	0.2	0.76	9.0	7.24	89.0
	0.3	0.62	7.0	7.25	89.0

The results of a typical count are given below. The media contained 0.3 per cent peptone. To one set β alanin was also added.

MEDIUM	WITHOUT NICOTINIC ACID; COUNT PER ML. AFTER HOURS 0	2	4	6	8	WITH NICOTINIC ACID (10γ/ML.) COUNT PER ML. AFTER HOURS: 0	2	4	6	8
Stock exper. medium.........	50	75	87	590	4,000	64	111	242	1,690	8,600
Stock + 10γ/ml. β alanin.....		43	127	720	4,500		72	247	1,650	8,900

Influence of nicotinic acid on the utilization of glucose by various species of dysentery bacilli (other than Shiga)

In the preliminary tests in the solid medium the Flexner strain used behaved in the same manner as did the Shiga strain. It was of interest, however, to note whether all types of dysentery

bacteria require nicotinic acid. The procedure was the same as in the preceding experiments. The medium contained 0.3 per cent peptone and 0.1 per cent glucose. The cultures were incubated for 48 hours at 37°C. and the glucose content determined. The results are shown in table 4. It will be noted that all of the type cultures used with the exception of two strains of Flexner failed to ferment glucose in the absence of nicotinic acid. The two strains which did not require nicotinic acid, fermented mannitol and maltose and were agglutinated to the full titre by a specific Flexner serum. The tests were repeated in liquid and solid media with the same results. It would seem, therefore,

TABLE 4

Influence of nicotinic acid on the fermentation of glucose by various dysentery bacteria (incubation 48 hours at 37°C.)

Strain	Glucose remaining in 9 ml. of media:				Growth in solid media	
	With nicotinic acid		Without nicotinic acid		With N acid	Without N acid
	mgm.	*per cent*	*mgm.*	*per cent*		
Flexner	0.12	1.0	7.88	93.0	+	−
Flexner x	0.30	3.0	7.75	91.0	+	−
Flexner z	0.20	2.0	0.35	3.0	+	+
Flexner w	0.0	0.0	0.2	2.0	+	+
Strong	0.5	7.0	6.84	81.0	+	−
Hiss-Y	0.64	8.0	7.44	89.0	+	−
Schmitz	0.70	8.0	7.80	93.0	+	0

that the Flexner strains vary in their need of nicotinic acid and represent in this respect an intermediate or transitional stage in the loss of ability to produce the cozymase. Those strains which corresponded with the Shiga type gave the same results: in the absence of nicotinic acid a small, limited, portion of the glucose disappeared but the process always stopped at the same point.

Influence of nicotinic acid on glucose fermentation by S. paratyphi A.

The preliminary tests on the solid semi-synthetic medium indicated that paratyphoid A differs from the other typhoid-paratyphoid bacteria in its inability to ferment glucose in the absence of nicotinic acid. The following are the results of a typical

analysis in the liquid medium containing 0.3 per cent peptone and 0.1 per cent glucose. The period of incubation was forty-eight hours at 37°C.

	WITHOUT NICOTINIC ACID, GLUCOSE REMAINING		WITH NICOTINIC ACID, GLUCOSE REMAINING	
	mgm.	*per cent*	*mgm.*	*per cent*
E. typhi 441	1.14	13.0	0.35	4.0
S. paratyphi A	6.64	79.0	0.64	7.0

An interesting problem was raised by the fact that in the medium used in these experiments, even when nicotinic acid was added and the sugar was practically completely utilized, no gas was formed. At first it was assumed that another substance was lacking in our medium which was present in nutrient broth—a carboxylase, which the organism could not synthesize. However, a variety of experiments showed that the controlling factor was the temperature of incubation. When the culture was grown in fermentation tubes in nutrient glucose broth and duplicate tubes incubated at 30 and 37°C. respectively, only a small amount of gas was produced at 37°C. and at least four to five times as much at 30°C. In the synthetic medium the differences were even more marked; as a rule no gas appeared at 37°C., while there was an appreciable amount at 30°C.

Table 5 summarizes the results of several experiments. The medium contained 0.3 per cent peptone and 0.2 per cent glucose. The incubation time was 48 hours. The inocula, etc. in the tubes incubated at the different temperatures were always identical.

The addition of β-alanin, d-alanin and B_1, singly and in combination, did not alter the results.

In order to rule out the possibility that these results were only apparent, due to a greater solubility of the CO_2 at 37°C., we determined the glucose consumed and the lactic acid produced at the two temperatures. The analyses were as a rule made in media containing 0.3 per cent peptone and 0.2 per cent glucose. For comparison, analyses were also made in media containing only 0.1 per cent glucose. The average results of a large series of experiments are summarized in table 6.

It will be noted that while 0.1 per cent glucose (1 mgm./ml.) is completely used up at both temperatures, the amount of lactic acid produced at 37°C., is one and a half times that at 30°C. When 0.2 per cent glucose is used, more sugar is consumed at 30°C. than at 37°C., with more acid produced at the latter temperature; in this case also, the amount of lactic acid produced at 37°C. is about 50 per cent greater than at 30°C.

As in the previous experiments, a certain amount of glucose is used up in the nicotinic-acid-free medium. The amount used is

TABLE 5

Gas production in the nicotinic acid medium in fermentation tubes incubated at 30 and 37°C. respectively

TEMP. OF INCUBATION	EXPER. MEDIUM	WITH NICOTINIC ACID	WITHOUT NICOTINIC ACID
°C.			
30	1	Growth good, 1.1 ml. gas	Growth poor, no acid, no gas
	2	2.0 ml. gas	Same as one
	3	0.9 ml. gas	Same as one
	4	0.6 ml. gas	
	Broth	3.0 ml. gas	3.0 ml. gas
	Broth	3.1 ml. gas	3.1 ml. gas
	Broth	2.1 ml. gas	
37	1	Growth good, acid, no gas	Growth good, no acid, no gas
	2	Growth good, 0.3 ml. gas	Growth good, no acid, no gas
	3	Growth good, acid, no gas	Growth good, no acid, no gas
	4	Growth good, acid, no gas	Growth good, no acid, no gas
	Broth	0.5 ml. gas	0.5 ml. gas
	Broth	0.8 ml. gas	0.8 ml. gas
	Broth	0.5 ml. gas	

small and is the same at both temperatures. It may well be that in addition to glucose fermentation, the organisms tested are also capable of a limited direct attack on the glucose molecule. This, however, is a matter for future investigation.

The only other organism of this group which showed this peculiar effect of temperature was Paratyphoid C. This organism does not require nicotinic acid. On the other hand, it uses up more sugar and produces more gas at 30°C. than it does at 37°C. No differences, however, were found in the quantities of

lactic acid produced. The average results of a series of experiments in our semi-synthetic medium containing 0.3 per cent glucose are summarized below. There were, of course, differ-

TABLE 6

Glucose consumed and lactic acid produced by S. paratyphi A. at 30 and 37°C. respectively; incubation period 48 hours

CONCENTRATION OF SUGAR	30°C.				37°C.			
	Glucose consumed; mgm./10 ml.		Lactic acid prod.; mgm./10 ml.		Glucose consumed; mgm./10 ml.		Lactic acid prod.; mgm./10 ml.	
	Nic.+	Nic.−	Nic. +	Nic.−	Nic.+	Nic.−	Nic.+	Nic.−
per cent								
0.1	Complete	0.65	1.23		Complete	1.21	2.00	
0.2	15.50	1.25	3.21	0.24	13.40	1.32	4.54	0.26

ences between individual experiments, but the trend was always the same and the averages represent the characteristic findings:

TEMPERATURE	SUGAR CONSUMED; MGM./10 ML.	LACTIC ACID PRODUCED; MGM./10	GAS PRODUCED; ML.
°C.			
30	21.27	3.43	2.3
37	17.82	3.37	1.6

It will be noted that the temperature effect is not as marked as in the case of Paratyphoid A; nevertheless, there is a distinct difference in the rate of fermentation at the two temperatures.

These results indicate that there is a new factor influencing the fermentation of glucose by these paratyphoid organisms which has not hitherto been taken into account. This effect is most marked in Paratyphoid A. It is difficult to explain how the temperature inhibits the further breakdown of the intermediate products of glucose fermentation, but the results are quite clear and consistent. At 37°C. the fermentation proceeds to the acid stage and the glycolysis continues until the accumulated acid inhibits further growth. At 30°C., on the other hand, the acid products are more readily broken down to gas, growth proceeds further and more sugar is utilized than at 37°C. This accounts

also for the fact that in the medium used growth was always better at 30°C. than at 37°C.

DISCUSSION

The results presented in this paper indicate that there are aspects of carbohydrate fermentation by bacteria which still require elucidation. Even in the colon-typhoid group of bacteria, the representatives of which have been extensively studied, new, fundamental differences have been found to exist in the enzymic apparatus of the various species. These differences have hitherto been obscured by the presence of an accessory substance in the nutritive medium used. It is conceivable that some of the contradictory results regarding the ability of this or that organism to ferment a given carbohydrate or to produce gas, were due to the variable concentrations of accessory substances in different batches of media, different conditions of incubation or some other as yet undefined factor.

The data presented above show that not all the species of the colon-typhoid-dysentery bacteria are possessed of a complete enzymic apparatus for the utilization of sugar. The colon bacilli, *Eberthella typhosa* and *Salmonella paratyphi B.* can ferment glucose in a semi-synthetic medium which does not contain nicotinic acid. On the other hand, *Salmonella paratyphi A.* and the various strains of dysentery bacilli cannot ferment glucose in this medium unless nicotinic acid is added. Nutrient broth evidently contains nicotinic acid, because the addition of a small quantity of broth to our medium has the same effect as nicotinic acid. Another fundamental difference is thus found to exist between the two types of paratyphoid, A. and B. An additional simple means of differentiation is thus made available.

Of special interest is the observation of the effect of temperature on the further breakdown of lactic or some other acid in glucose fermentation. This phenomenon has been observed in the case of paratyphoid A and C, respectively, but not in the other strains of paratyphoid or in the coli strains tested.

We have not been able to elucidate the nature of the inhibition produced by temperature. The analyses indicate that with paratyphoid A. the failure to produce gas is accompanied by an

accumulation of lactic acid. It would seem, therefore, that the higher temperature inhibits the action of an enzyme, probably of the nature of a carboxylase. The fact that temperature can exert such an influence has apparently hitherto been overlooked.

Apart from the general interest these observations may have in relation to carbohydrate fermentation by bacteria, it may well be that the deterioration of the enzymic system of these organisms may have some bearing on their specific parasitism and invasiveness. The whole question requires re-investigation in the light of the newer knowledge of the relation of accessory substances to the physiologic activity of bacteria.

SUMMARY

Salmonella paratyphi A. and the various species of *Shigella dysenteriae* are unable to ferment glucose in semi-synthetic media which do not contain nictotinic acid. The Flexner strains are variable in this respect.

A high incubation temperature (37°C.) inhibits partially or wholly, according to the nature of the medium, the production of gas by *Salmonella paratyphi A.* and *C.* respectively, but not by other species of paratyphoid or coli bacilli tested.

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A COLLODION SAC FOR USE IN ANIMAL EXPERIMENTATION[1]

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Sanarelli (1891) is believed to have been the first to employ collodion sacs in animal experimentation. Subsequently, sacs of different types have been used in various *in vivo* experiments by a number of investigators (see bibliography). Alcohol-ether collodion was employed in each case for the preparation of the semipermeable membranes. In order to withstand the distorting effect of the intestinal movements, the collodion had to be of rather high concentration, with correspondingly low permeability. In undertaking the present work, it was felt that certain investigations could be made much more satisfactorily if sacs could be prepared that were more permeable and at the same time more rugged than those that had been previously described. According to Elford (1931), acetic-acid collodion membranes are not only of a higher order of permeability than those of alcohol-ether collodion, but they are also highly heteroporous. The use of this fragile material necessitated the finding of a practical support. Fouard (1909) impregnated wire gauze with collodion. This type of support was tried and was found to be highly practical. The description by Gates (1921) was used as the point of departure in trying to prepare a highly permeable yet sturdy modification of his type of sac. After a considerable number of failures, a satisfactory sac has been evolved which is quite different from that of Gates. However, many of his practices, particularly those concerning the handling of sacs, have been incorporated.

[1] Presented before Eastern New York Branch of the Society of American Bacteriologists, December 2, 1938, Albany, New York.

In brief, the sac consists of a semipermeable membrane supported by a cylinder of stainless-steel wire gauze closed at one end with molded carnauba wax and capped at the other end also by wax, through which a glass tube passes. The technic of manufacturing the framework is an empirical one, the detailed procedures having been adopted after trial and error.[2]

MATERIALS AND METHODS

Materials

The equipment used in making the framework of the sac is displayed in plate 1. Carnauba wax is melted in a glass beaker, ready to be pipetted to the two glass molds. The first mold consists of 9-mm. glass tubing that widens at the top to form a glass cup 2 cm. in diameter and 1.5 cm. high. The tube is filled with melted paraffin to within 2.5 cm. of the bottom of the cup. The paraffin hardens and forms a permanent plug. The mold thus prepared is held in a vertical position by means of clamps. The second mold, of the same dimensions as the cup of the first, is made by severing the bottom part of a test tube with a hot wire. The tubes that form the openings into the collodion sacs are made of 6-mm. glass tubing cut into 3-cm. lengths and fire-polished. Rectangles of 100-mesh stainless-steel wire gauze, 4 by 3 cm., to be used as supports for the semipermeable membranes are scrupulously cleaned in boiling 25 per cent sodium hydroxide, rinsed in water, dipped in acetone, and dried in air. They are curved into cylinders with the long dimension as circumference.

Manufacture of framework

Before pipetting the wax into the molds, the inner surfaces are wiped with vaseline to prevent the wax from adhering at any point. One of the 3-cm. glass tubes is lowered into the first mold so that one end rests on the paraffin plug and the other projects up into the glass cup. Enough water is now delivered into the tube portion of the mold to fill it up to the bottom of the cup. Next, carnauba wax is allowed to run into the mold from a pipette

[2] The work was done with the assistance of Charles A. Clark.

until it all but reaches the top of the 3-cm. glass tube. It will not run down into the tube portion of the mold for it is lighter than the water there. A wire-gauze cylinder is immediately lowered into the cooling wax, which, solidifying rapidly, contracts so that the approximating edges of the gauze are pressed tightly together. After cooling, the wax is eased out of the mold, together with the attached glass tube and gauze cylinder, as shown in plate 2.

Using the second mold, the wax bottom of the framework is affixed in a comparable manner. The wax is shaped further by means of a hot spatula so that the edges become smooth and almost flush with the gauze cylinder. Although it does not tend to spatter during this procedure, provided it is dry, care must be taken to prevent wax from running down the gauze lest the future dialyzing surface of the sac be unnecessarily reduced. The line of approximation of the gauze is sealed by allowing melted wax to flow into the adjacent interstices from a capillary pipette. An identification number is printed on the wax of each frame in indelible ink. The frames are now ready to be reinforced with heavy alcohol-ether collodion; the percentage of nitrocellulose in this collodion is immaterial as long as it just flows smoothly. To prevent the collodion from running up into the short glass tube when the inverted sac is dipped, the tip of the tube is first touched to the collodion and then set aside for a short time to dry. During this time the bottom of the sac can be coated by lowering it into a jar of heavy collodion until the junction of the wax and wire gauze is barely submerged. After the excess collodion has drained off, it is allowed to dry. The upper end of the sac is coated in the same manner, the 3-cm. glass tube being completely submerged during the process. Using a scalpel, the distal portion of the 3-cm. tube is bared of collodion. Foreign material clogging any of the interstices of the gauze is flicked out with the point of the scalpel or a needle.

Semipermeable membrane

After several hours, when the heavy alcohol-ether collodion has become thoroughly hard but not so dry that it is very brittle or

tends to peel, the sac is dipped into acetic-acid collodion of whatever strength is required. Tests have been performed with sacs made with from 4.5 to 10.3 per cent Parlodion, by weight, in glacial acetic acid. A description of the experiments for which collodion solutions of different strengths have been used is not germane to this article, but it may be said that the higher concentrations of collodion have proved to be practical and of greater usefulness. The handling of the sac is facilitated by the use of a short piece of rubber tubing drawn through the hole of a no. 5 rubber stopper so that it projects a short distance from each end. The rubber tubing is of adequate size to hold the glass tube lightly but firmly. After the sac has been attached, it is lowered into the acetic-acid collodion in a wide-mouthed bottle. If the collodion is too cool, it will not cover the gauze smoothly. Slight rotation of the sac serves to dispel any bubbles which may cling to the surface of the gauze. As soon as it has been submerged up to the lower portion of the 3-cm. tube, it is slowly withdrawn. The excess collodion is allowed to drip into a Petri dish. While the sac is submerged, the collodion flows through the interstices and down the inner surface of the gauze. As it collects on the bottom, it is aspirated by a special capillary pipette, depicted in plate 3. The drawn-out end of the pipette must be large enough to allow the collodion to be aspirated without clogging, yet small enough to allow plenty of air space around it when it is lowered through the rubber and glass tubing to the bottom of the sac; otherwise the negative pressure established in the sac may rupture the membrane. Aspiration must be controlled so that collodion and not air is sucked up; the drawing of a vigorous stream of air into the sac is to be avoided. Frequent flushing of the pipette with glacial acetic acid helps to prevent clogging. After the sac has drained for an arbitrary but constant period (60 seconds), it is plunged into a bottle of stone-filtered tap water. The aspirating is continued, however, until the collodion on the inside bottom has been removed as completely as possible. A stiff wire bent into the shape of a hairpin is slid under the rubber stopper and allowed to rest on the edges of the bottle, thus suspending the sac in the water. The sac is filled with water by

means of a capillary pipette. The water is changed about every half hour or hour, for from four to six hours, the water on the inside being changed at the same time. A longitudinal section of the completed sac is schematized in figure 1.

Testing the sac

The method of testing porosity and intactness was suggested by the work of Asheshov (1933). The sac and attached rubber tubing are filled to overflowing with filtered tap water and a

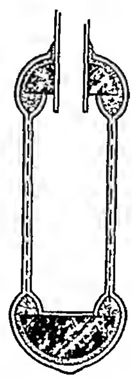

FIG. 1

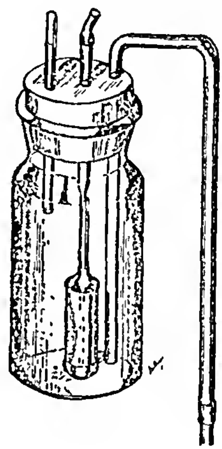

FIG. 2

FIG. 1. SCHEMATIC DIAGRAM OF SAC

The glass tube at the top of the sac passes through the wax cap, represented by shading. The wire gauze is indicated by interrupted lines. The heavy stippling reveals the portion of the framework covered by heavy alcohol-ether collodion and the light stippling shows coverage by the acetic-acid collodion.

FIG. 2. RINSING BOTTLE

filled 10-ml. pipette is thrust firmly into the upper end of the rubber tubing. The pipette is held in a vertical position, as shown in plate 4, and the level of the water in which the sac is suspended is adjusted so that for each test it is the same distance (2 cm.) above the upper margin of the semipermeable membrane. The length of time required for the meniscus to descend from the 0-ml. to the 1-ml. mark is recorded. The temperature of the room and the barometric pressure are noted. Any marked increase in speed of filtration above the average for the percentage

of collodion used generally indicates the presence of a flaw in the sac. As a further check on the intactness of the sac, it is withdrawn from the water with the pipette still attached and the surface gently brushed with filter paper. If drops immediately and persistently appear at any one spot after each brushing, the presence of a flaw is indicated and the sac is discarded.

Sterilizing the sac

The sac is sterilized in "B.K." solution,[3] 1 part in 100 of distilled water, for about eighteen hours at room temperature. Except for spore-forming bacilli, which on rare occasions have subsequently proliferated, contaminating bacteria are always destroyed by this method. The sterilizing solution is then washed out with 3 liters of sterile distilled water by means of the sterile rinsing device shown in plate 5. After the stoppers are inserted in the bottles, they may be swathed in cotton soaked in cresol solution as a precaution against contamination. The water is delivered by syphon to the rinsing bottle which is diagrammed in figure 2. The water flows into the sac through the water inlet tube and the capillary tube, spills out of the mouth of the sac, runs down the outside, and causes the water level in the bottle to rise slowly until it reaches the cotton-plugged air vent. If the stopper is tightly inserted in the bottle, the water will not reach its under surface but will rise in the vent and at the same time spill over through the outlet tube, establishing a syphon which, because of the large size of the outlet tube, promptly drains the bottle; thus the syphon is broken. The continuous, fine stream of water entering through the capillary tube now causes the water level to rise again, and the automatic rinsing continues. The water inlet tube may be clamped off from time to time, thus allowing the sac to soak. It has been found that the syphon will break more readily if the inner surface of the outlet tube is coated once with a thin layer of paraffin.

[3] A sodium hypochlorite solution marketed by the Pennsylvania Salt Manufacturing Company of Philadelphia. Quigley and Sickles (J. Bact., 1937, 33, 110-111) recommended it for sterilizing collodion membranes.

Filling the sac

A test-tube holder fitted with extra strands of wire and sterilized in boiling water serves as a satisfactory tool for handling the sterile sac. The water is expelled from the inverted sac by introducing into it a gentle stream of air through a cotton-plugged sterile capillary pipette. A flat-bottomed glass cup, made from a small vial by cutting off the upper portion with a hot wire, serves to hold the sac from this point on. Before use, the cup is put, open end first, into a large test tube and sterilized. In order to introduce the sac, the test tube is inverted and the cotton plug and the cup are withdrawn together. The sac is then deposited in the cup, and the cup and plug are thrust back into the inverted test tube as shown in plate 6. The sac is allowed to remain empty for only a short time, lest drying alter the permeability. When the sac is to be filled, the cup is removed again, and the glass tube of the sac thoroughly dried in a Bunsen flame, care being taken not to crack it by too rapid heating. The test fluid is introduced as shown in plate 7. Great caution must be exercised to prevent any fluid from running down the outside of the glass tube; as an added precaution, the tube is thoroughly flamed again after the pipette has been withdrawn. A few drops of sterile, melted paraffin serve to plug the glass tube. A coating of alcohol-ether collodion is applied to the outside of the tube; thus the whole surface of the sac is sealed with collodion.

Implantation in animal

The sac is now ready to be introduced into the peritoneal cavity of the experimental animal. The operative technic need not be described here. When the sac is removed at the conclusion of the experiment, a hot wire is thrust through the collodion and paraffin, thus providing a hole through which the contents can be aspirated with a Pasteur pipette. Plate 8 shows two sacs after their removal from an experimental animal. The wall of one is clean and the wire mesh is plainly visible through the film of collodion. The other sac is covered with a heavy coating of fibrin.

The results of experiments entailing the use of the described collodion sacs will be published subsequently.

SUMMARY

A review is presented of the development of the collodion-sac technic for use in animal experimentation.

A method of making a sturdy, yet permeable, sac is detailed.

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PLATE 1

Materials Used in Making the Framework of Sacs

PLATE 2

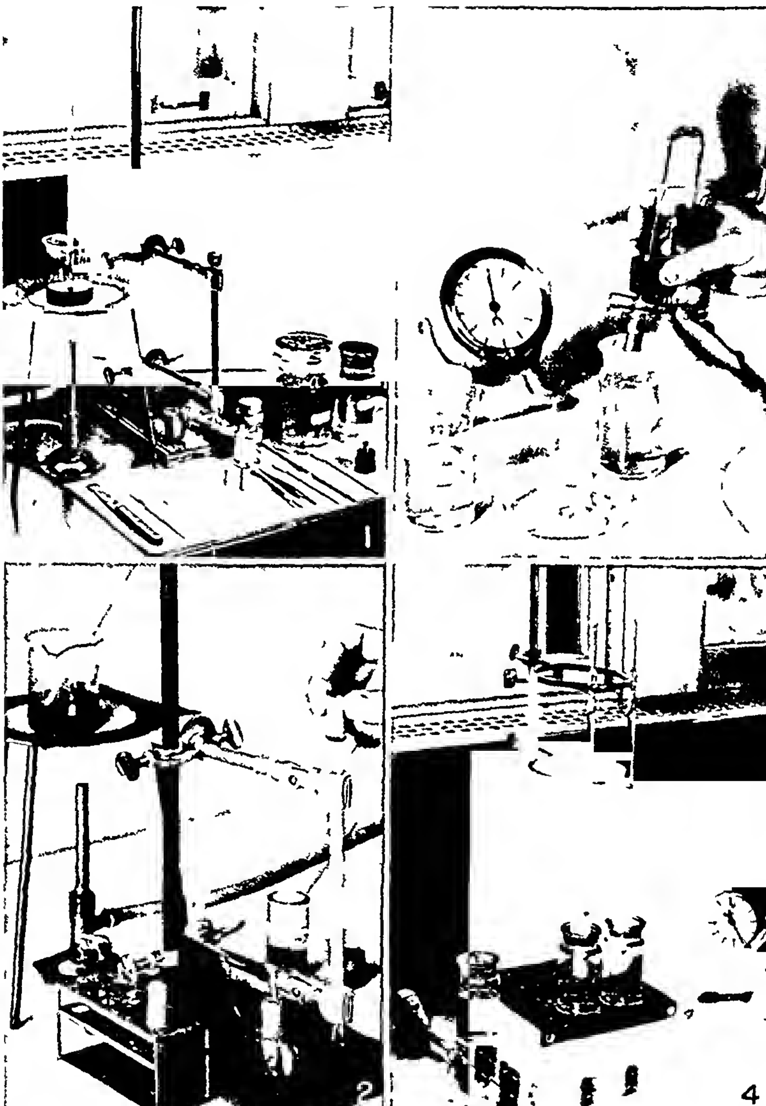
Removing a Partly Finished Sac from the First Mold

PLATE 3

Aspirating Excess Collodion in a Sac, and Gelling the Collodion in Water

PLATE 4

Testing the Rate of Water Flow through Sacs



(A H. Harris Collodion Sac for Animal Experimentation)

PLATE 5

Rinsing Out the Sterilizing Solution

PLATE 6

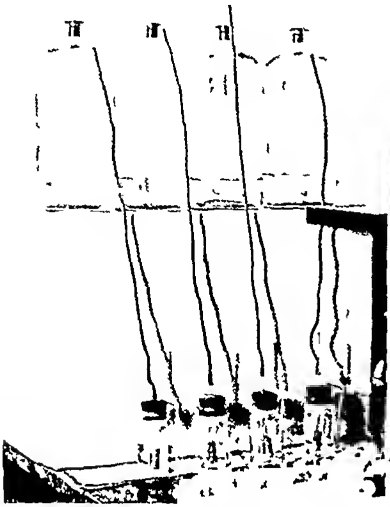
Pasteur Pipettes, and Sacs Ready to Be Filled

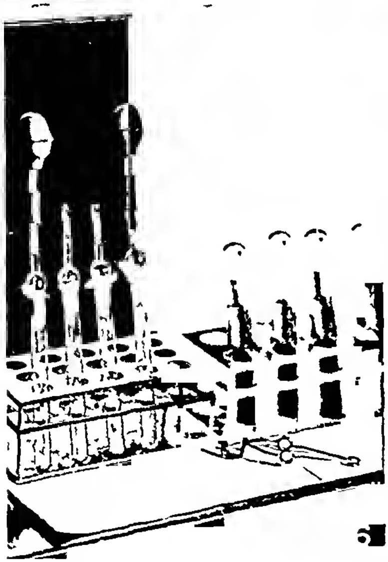
PLATE 7

Filling a Sac

PLATE 8

Sacs after Removal from the Peritoneal Cavity of a Rabbit. Note Fibrin Deposition on One





(A. H. Harris: Collodion Sac for Animal Experimentation)

STUDIES ON THE MODE OF ACTION OF SULFANILAMIDE IN VITRO

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Recently Lockwood (1938a and b) demonstrated that sulfanilamide in concentrations similar to those occurring in sulfanilamide-treated patients is markedly bactericidal for virulent streptococci growing in peptone-free human serum, but is merely bacteriostatic when even small amounts of peptone are present in the serum.

In this paper we have attempted to repeat and extend Lockwood's work using rabbit instead of human serum as a culture medium for the streptococcus.

METHODS AND MATERIALS

Strain used

Only one strain, C 203 (Group A) was employed in this work (this is one of the two strains that Lockwood used). Its virulence was maintained by passage through mice once in every two weeks. The streptococcus was grown in 5 per cent sheep-blood meat-infusion broth for 14 hours at 37.5°C., was standardized, and was kept in the ice box. In a few experiments a 3-hour culture of the organism in neo-peptone broth containing an equal amount of rabbit serum was used.

Source of blood

The rabbit serum was obtained fresh for each experiment by intracardial puncture. In all, 16 rabbits of 2000 to 3000 grams were used. Our strain C 203 appeared to grow equally well in the sera from all these rabbits. When a large quantity of serum

was needed for any one experiment, the sera from several rabbits were pooled.

In our first experiments we used serum from defibrinated blood which contained small amounts of hemoglobin. Later we found that serum from the clot was just as suitable a medium as the defibrinated serum for our work and used this in our subsequent tests.

Sulfanilamide solutions

Sulfanilamide (Prontylin-Winthrop Chemical Co.) solutions were made up in appropriate dilutions in physiological salt solution.

Procedure of the tests

To a series of small test tubes (10 x 100 mm.) containing 1 ml. each of rabbit serum were pipetted in order named and well mixed: 0.05 ml. of 10 per cent neopeptone when desired, 0.05 ml. of the appropriate concentration of sulfanilamide solution, physiological salt solution in the amount needed to bring up all tubes to equal volume and 0.1 ml. of the culture dilution. The tubes were then placed at the temperatures required in the experiment. (In these experiments no apparatus for the continuous mixing of contents of the tubes was used.)

To obtain the required number of organisms in 0.1 ml. volume for these tests, the culture of streptococcus was serially diluted in broth for all but the final two dilutions which were made in rabbit serum diluted 1:10 in saline. Broth instead of saline was employed because of the findings that not more than one half the calculated number of organisms grew into colonies and many of these colonies developed extremely slowly, (being visible on blood plates only after 48 hours at 37.5°C.), when this strain of streptococcus was exposed to physiological salt solution during the process of dilution preliminary to seeding the test cultures. When dilutions are made in broth or in rabbit serum diluted 1:10 in saline, the final counts check accurately with those expected from the use of the standardized culture and the colonies are all approximately the same in size and in extent of hemolysis.

The last two dilutions were made in 1:10 rabbit serum in order to eliminate all but a trace of peptone present in the diluent broth (approximately 0.01 mgm. peptone per ml.).

At the start of each experiment, 0.1 ml. of the streptococcus dilution to be used, further diluted in broth if necessary for accurate counts, was always plated out in duplicate and the average of both counts taken for the first counts in the figures. Bacterial counts of the test cultures were made by the dilution plate method. Cultures to be counted were diluted with broth, a fresh pipette being used for each dilution. To calculate any one count, an average of all countable plates was taken.

In the figures, 0 signifies no growth from the inoculation of 0.05 ml. or 0.1 ml. of test materials in blood plates. No effort was made to prove absolute sterility in most experiments.

Smears

Smears were made routinely and stained with Wright's stain at the same time that the test materials were plated for bacterial counts. Our observations on the effect of sulfanilamide on the morphology of the streptococcus growing in rabbit serum are in accord with those of Lockwood on the morphological changes of organisms growing in human serum under the influence of sulfanilamide. Morphological alterations consisting of long chains, chains with missing cells, persistence of pink capsules, increase in size of individual cells which were often irregular and coalesced together, and degenerating forms, were the characteristic changes seen. Degenerating forms were usually only noticed when the organisms were dying out. We found, as Lockwood did, that morphological changes were evident even at concentrations of sulfanilamide which had only questionable bacteriostatic effects.

EXPERIMENTAL

In our preliminary experiments we found that when peptone-free rabbit serum is inoculated with 3000 organisms per ml., even 1:1000 dilution of sulfanilamide (1 mgm. per ml.) is rarely bactericidal though it is definitely bacteriostatic, and sulfanilamide in 1:5000 and 1:10,000 dilution (0.2 and 0.1 mgm. re-

spectively, per ml.) is generally merely somewhat bacteriostatic. When serum containing peptone (5 mgm. per ml.) instead of peptone-free serum is employed, sulfanilamide is even less effective but the difference in growth in the two media containing the same concentrations of sulfanilamide is not striking. In figure

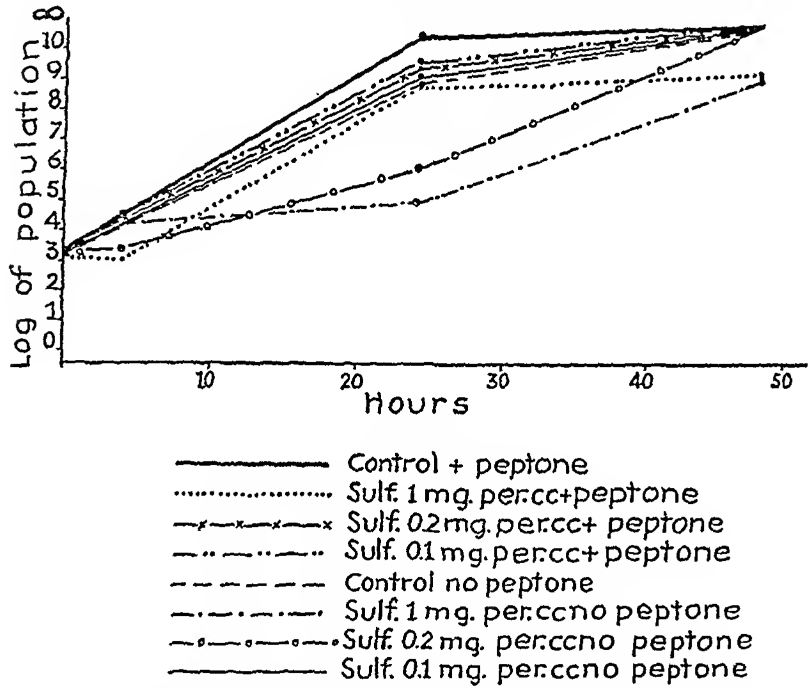

FIG. 1. EFFECT OF SULFANILAMIDE ON THE GROWTH OF THE STREPTOCOCCUS AT 37.5°C. IN FRESH RABBIT SERUM WITH AND WITHOUT PEPTONE AFTER A RELATIVELY LARGE INOCULUM (3000 ORGANISMS PER ML.)

Composite of 4 different experiments.

These experiments show the lack of any bacteriostatic effect of sulfanilamide in 1, 0.2 and 0.1 mgm. per cc. concentrations on the growth of the streptococcus in rabbit serum containing peptone and only a slight bacteriostatic effect of the drug in the same concentrations on organisms growing in peptone-free serum.

1 are presented the composite results of 4 different experiments on the effect of sulfanilamide on the growth of the streptococcus on rabbit serum with and without neopeptone at 37.5°C. which bring out these points.

Experiments such as these are in marked contrast to those of Lockwood in which the effect of sulfanilamide on the growth of

the streptococcus on human serum with and without neopeptone was tested. In his experiments, sulfanilamide in a dilution of 1:10,000 (0.1 mgm. per ml.) killed 3000 organisms or chains growing in peptone-free serum in 24 hours at 37.5°C., whereas in the presence of peptone, this drug in the same concentration was merely bacteriostatic.

In seeking an explanation for the lack of bactericidal properties of sulfanimamide on streptococci growing in rabbit serum, we compared the speed of growth of the organism on rabbit and on human sera with and without neopeptone and noted the following differences in the rate of growth in these 4 media: In the first place, during the first 20 hours at 37.5°C. with a 3000-organism inoculum, when peptone is present, rabbit serum was found to be a much better medium for growth of the streptococcus than human serum. Secondly, in human serum, the organisms multiplied much faster in serum containing peptone than in peptone-free serum, whereas in rabbit serum the organisms grew almost as profusely when peptone was excluded from the medium as when it was not.

From these observations, it seemed worth inquiring whether these two facts noted above; one, the greater rate of multiplication in rabbit serum as compared to that in human serum; and two, the fact that the streptococcus after an inoculation of 3000 organisms grows almost equally well in rabbit serum whether it contains peptone or not, while it grows relatively much faster in human serum with peptone than in peptone-free human serum, might account for our inability to demonstrate bactericidal properties of sulfanilamide in rabbit serum. Accordingly we devised experiments to retard the rate of multiplication of the organisms in rabbit serum with the thought that thus we might obtain results with sulfanilamide comparable to those of Lockwood using human serum.

Effect of sulfanilamide on the streptococcus growing in rabbit serum with and without peptone, when the rate of growth is retarded

1. By the use of small inocula. Since it has been demonstrated by many investigators that growth of the streptococcus must

proceed for several hours in the presence of sulfanilamide before the activity of this drug is demonstrable, our poor results with sulfanilamide on the growth of the organism in rabbit serum as compared to that in human serum seemed at least partially explicable on the ground that after several hours of growth many more organisms are present in the rabbit serum than in the human serum on which the drug would be acting. Hence, it was thought that by using a small inoculum (400 organisms per ml.) the bacterial concentration in rabbit serum after the first few hours of incubation would be much reduced as compared to that following a much larger inoculum (3000 organisms) and that this growth (after a small inoculation) would be more similar to the growth present in human serum after the same number of hours of growth following the larger inoculum (3000 organisms). Furthermore, it was thought that by the use of a small inoculum and consequently a slower growth of the organisms, a greater difference in the speed of multiplication might perhaps be noticeable between the growth in rabbit serum containing peptone and the growth in peptone-free rabbit serum, less growth being present in the latter. If this relationship were shown to exist in the case of rabbit serum, it would better simulate the relationship present in human serum with and without peptone and the striking action of sulfanilamide on the streptococcus growing in peptone-free human serum could in part be correlated with the slow multiplication of the organisms in this medium.

Accordingly, we set up experiments using a small inoculum and were successful in demonstrating a much more marked effect of sulfanilamide when the organisms were grown in peptone-free serum instead of serum containing peptone (see fig. 2). Moreover, these results appeared to be correlated with a slower growth in the control tubes of peptone-free serum than in the control tubes of serum containing peptone (not shown in this figure). (This experiment also shows that peptone-free rabbit serum does not sustain the growth of the streptococcus for more than 60 hours, whereas rabbit serum containing peptone is a good medium for growth for at least 76 hours).

2. By the growth of the organism at room temperature (20°C.).

It next occurred to us that the speed of growth of the streptococcus might be still further delayed and, thus, even better results with sulfanilamide obtained if the retardation effect of small inocula were combined with growth at a relatively low temperature (20°C.) instead of at 37.5°C. To our surprise, in

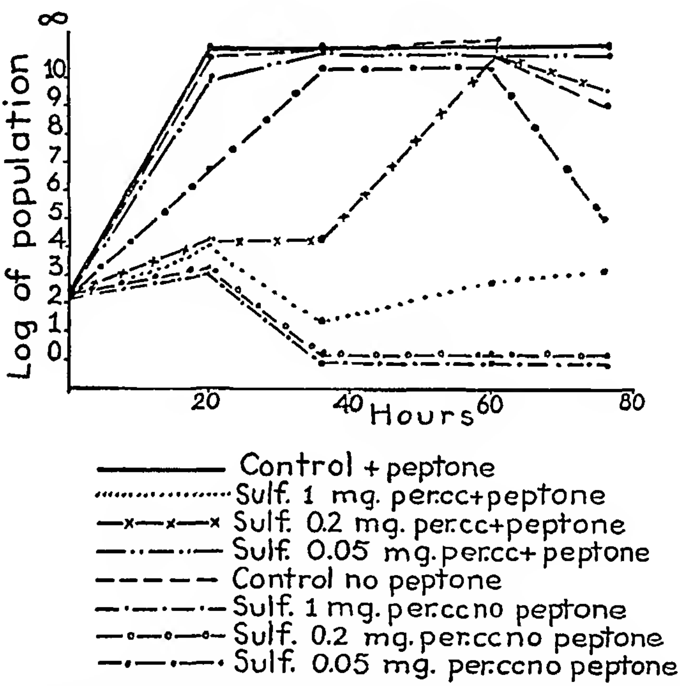

FIG. 2. EFFECT OF SULFANILAMIDE ON THE GROWTH OF THE STREPTOCOCCUS AT 37.5°C. IN FRESH RABBIT SERUM WITH AND WITHOUT PEPTONE AFTER A RELATIVELY SMALL INOCULUM (400 ORGANISMS PER ML.)

This experiment contrasts the bactericidal effect in 40 hours of sulfanilamide in concentrations of 1 and 0.2 mgm. per ml. on organisms growing in peptone-free rabbit serum with the merely bacteriostatic effect of the drug in the same concentrations when peptone is present in the medium.

these experiments although as expected, the organisms multiplied more slowly at 20°C. than at 37.5°C., the sulfanilamide had a negligible effect on the growth of the organisms even at a concentration of 1:1000 (1 mgm. per ml.) whether or not peptone was present in the medium. When growing at 20°C., the organ-

isms increased at a uniform rate for 48 hours and there was very little difference in the amount of growth whether or not peptone was present in the medium. In figure 3 is presented an experiment which demonstrates these points.

These last experiments in which it was shown that sulfanilamide is not active at 20°C., or only to a very slight extent, indicated that the temperature at which tests are carried out is of major

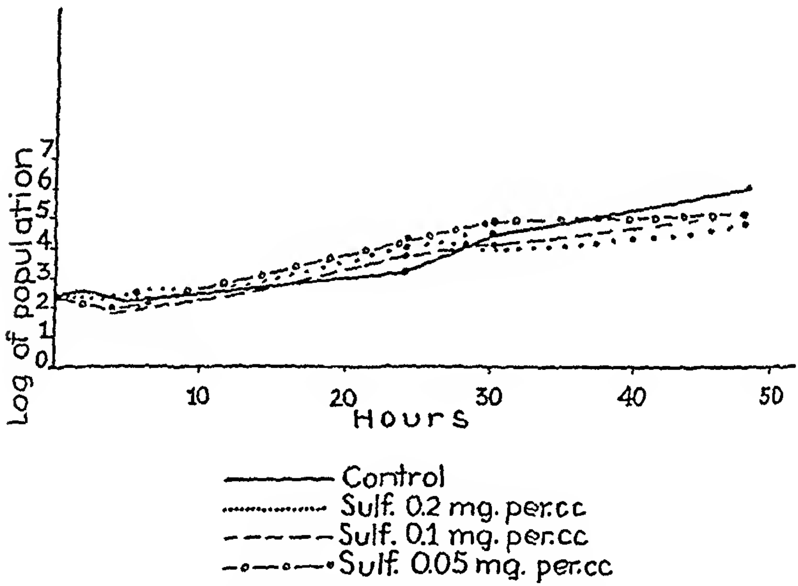

Fig. 3. Effect of Sulfanilamide on the Growth of the Streptococcus in Peptone-free Rabbit Serum at 20°C. after a Relatively Small Inoculum (400 Organisms per ml.)

This experiment shows that sulfanilamide has a negligible effect on the growth of the organisms in peptone-free rabbit serum. The curves showing the effect of sulfanilamide on the growth of the organism in serum containing peptone are practically identical with the curves shown above in fig. 3 and are omitted.

importance in the activity of sulfanilamide. They suggested that the activity of sulfanilamide, which is demonstrable at 37.5°C. might be further increased if the temperature at which the tests were carried out was elevated above this temperature. Furthermore, it was thought that carrying out tests for effectiveness of sulfanilamide at elevated temperatures, since such temperatures would not be optimum for the growth of this organism,

might have the additional advantage of retarding the growth of the organism, which retardation, as shown above, improves the bactericidal properties of sulfanilamide. The following sections deal with the investigation of these points.

White and Parker (1938) have just reported that sulfanilamide is more effective *in vitro* at 40°C. than at 37°C. It is interesting that these investigators came to this conclusion by a different method of approach from ours. They had previously found that many freshly isolated strains of hemolytic streptococcus, Group A, grew rapidly and abundantly at 40°C. and it therefore occurred to them to test the action of sulfanilamide at this temperature. Accordingly in experiments carried out to test this idea, they found that sulfanilamide in concentrations of 0.2 mgm. per ml. (20 mgm. per cent) had consistently striking bactericidal properties for these strains at 40°C. whereas at 37°C. sulfanilamide in the same concentration was only slightly bacteriostatic. This work was carried out on organisms growing in either defibrinated horse blood or in "P D" broth.

Effect of sulfanilamide on streptococci growing at 39°C. in rabbit serum with and without peptone

These experiments were carried out similarly to our previous ones except that the tests were incubated for various periods of time at 39°C. instead of at 37.5°C.

In regard to the control tests, we first found that when peptone is excluded from the medium and a small inoculum is used (400 organisms), the streptococci may first multiply slowly for 4½ hours but soon die out; at the end of 18 hours the cultures are sterile. On the other hand, when peptone is added to the medium in the usual quantity, the same inoculum will result in good growth, but the bacterial concentrations never quite achieve the level resulting from the incubation of the same number of organisms in the same medium at 37.5°C. (White and Parker also noted this fact with their strains of streptococcus.) The effect of sulfanilamide in tests set up with rabbit serum containing peptone inoculated with 400 organisms was markedly increased when the experiments were carried out at 39°C. instead of 37.5°C.,

the drug being bactericidal in 1:20,000 dilution (0.05 mgm. per ml.) in 18 hours at 39°C. whereas, as previously shown and confirmed many times, the drug was merely slightly bacteriostatic even in 1:5000 dilution (0.2 mgm. per ml.) at 37.5°C. In figure 4 is presented an experiment comparing the effect of sulfanilamide at 37.5°C. and at 39°C. on streptococci growing in rabbit serum

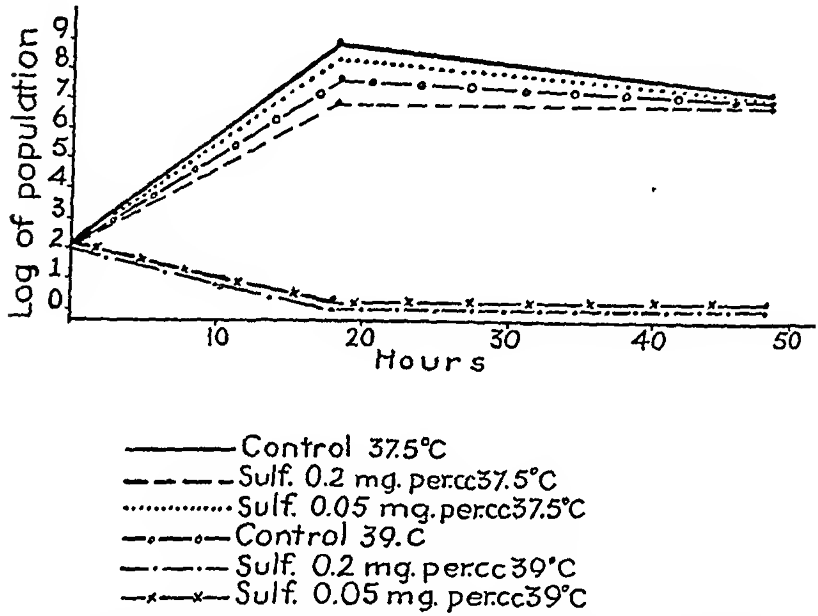

Fig. 4. Effect of Sulfanilamide on the Growth of the Streptococcus at 37.5°C. and at 39°C. in Fresh Rabbit Serum Containing Peptone after a Small Inoculum (300 Organisms per ml.)

This experiment contrasts the bactericidal effects of sulfanilamide at 39°C. in 18 hours in concentrations of 0.2 and 0.05 mgm. per milliliter with the merely slight bacteriostatic effects in the same concentrations of the drug at 37.5°C. In this experiment tests were also set up at 39°C. in which the inoculum contained 4000 and 1600 organisms resulting also in sterility in 18 hours in the tubes containing sulfanilamide.

containing peptone. This demonstrates the striking bactericidal effect of the drug at 39°C. and the total lack of such effect at 37.5°C. It also shows that the maximal bacterial population is slightly higher in the 37.5°C. control than in the 39°C. control.

No attempt was made to determine the minimal concentration of sulfanilamide that would be bactericidal for the streptococcus

at 39°C. (or at 40°C.). That a concentration of 0.05 mgm. per ml. of the drug is higher than that needed for bacteriolysis was demonstrated in one experiment in which 0.02 mgm. per ml. (1:50,000 dilution) was shown to be bactericidal in 18 hours at 39°C. after an initial inoculum of 4000 organisms per ml. growing in rabbit serum containing peptone.

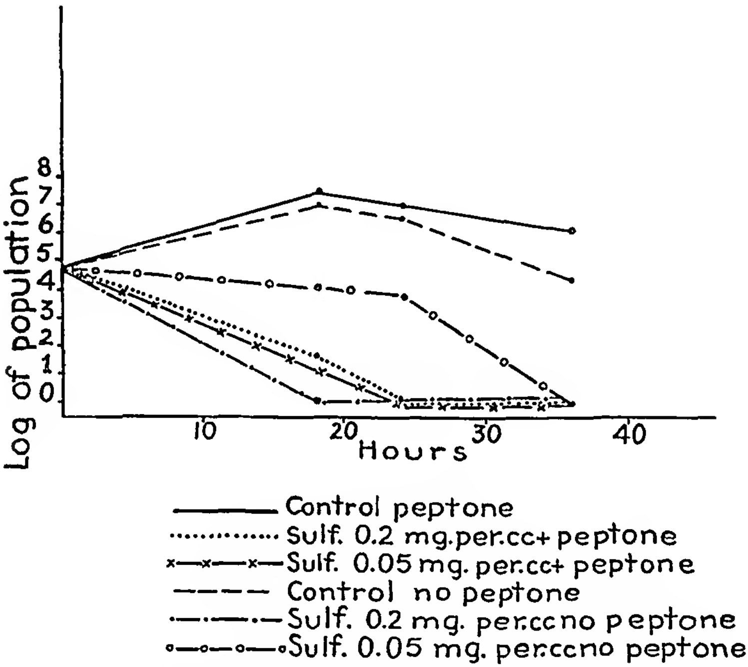

FIG. 5. EFFECT OF SULFANILAMIDE ON THE GROWTH OF THE STREPTOCOCCUS IN FRESH RABBIT SERUM WITH AND WITHOUT PEPTONE AT 39°C.

This experiment shows that at 39°C., sulfanilamide has if anything less bactericidal effect on organisms growing in peptone-free rabbit serum than in serum containing peptone.

In order to obtain data on the effect of sulfanilamide on organisms growing in peptone-free serum at 39°C., tests were set up in which the number of organisms in the inoculum was increased. In the control tubes, when the initial concentration of organisms was roughly 4000, the organisms did multiply progressively in peptone-free rabbit serum at 39°C., the maximal

bacterial concentration being reached after 20 hours incubation, after which time a continuous decrease in viable bacteria to sterility in 72 hours occurred. The highest bacterial population in the control tests containing peptone after the same inoculum, also reached at approximately 20 hours after inoculation, was

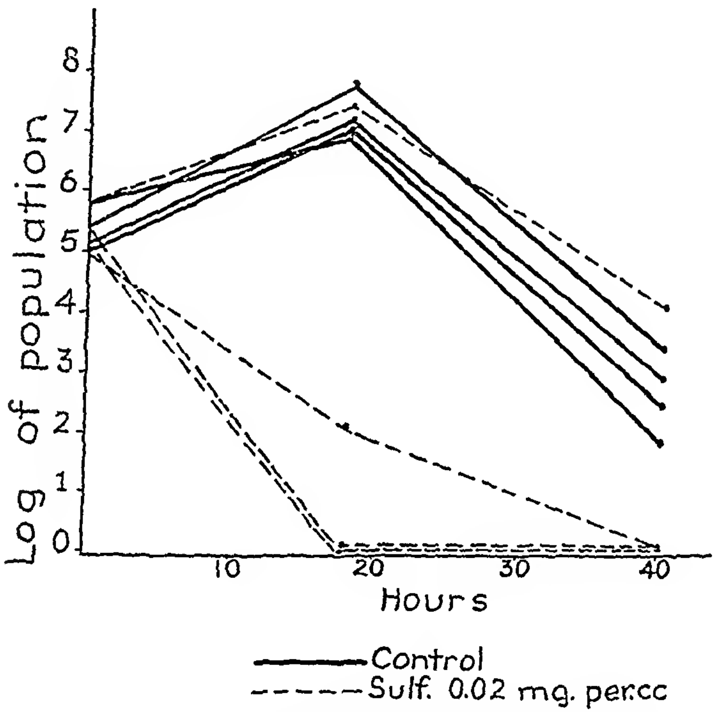

FIG. 6. EFFECT OF SULFANILAMIDE ON THE GROWTH OF THE STREPTOCOCCUS IN FRESH RABBIT SERUM CONTAINING PEPTONE AT 40°C.

This experiment shows that sulfanilamide is bactericidal in 1:50,000 dilution (0.02 mgm. per milliliter) at 40°C. on large numbers of streptococci growing in rabbit serum containing peptone. In this experiment a 3 hour 50 per cent neo peptone rabbit serum culture was used.

always slightly higher than that found in peptone-free serum, and in this medium the decrease in the viable organisms was delayed as compared with that of the control tests without peptone. However, surprisingly enough, in spite of the difference in the amount of growth in the 2 media just noted, the bactericidal action of sulfanilamide was more pronounced at 39°C. when

peptone was present in the medium than when it was not, a result directly contrary to that anticipated from our previous tests at 37.5°C. This last finding is demonstrated in figure 5 in which it is seen that sulfanilamide in 1:20,000 dilution (0.05 mgm. per ml.) is bactericidal in 24 hours after an inoculum of 80,000 organisms when peptone is present and bactericidal only in 36 hours when peptone is excluded from the medium. This type of experiment has been repeated several times, always with essentially the same results.

Effect of sulfanilamide on the growth of the streptococcus at 40°C. in rabbit serum with and without peptone

It was found impossible to produce progressive growth of our strain of streptococcus at 40°C. when peptone was excluded from the medium unless the inoculum used was very large. However, when the serum contained peptone the organisms grew well but the initial inoculum needed to produce growth was roughly 20,000 organisms. In figure 6 is presented an experiment showing that sulfanilamide in 1:50,000 (0.02 mgm. per ml.) is bactericidal at 40°C. after an inoculum of 400,000 organisms and is not bactericidal in 1:50,000 (0.02 mgm. per ml.) after an inoculum of 800,000 organisms.

DISCUSSION

On the basis of his finding that sulfanilamide is markedly more effective on streptococci growing in peptone-free human serum than in human serum containing peptone, Lockwood believes that "the evidence justifies the tentative conclusion that sulfanilamide interferes with the ability of hemolytic streptococci to use serum or tissue protein as food from which to obtain nitrogen." Our experiments seem to show that this is not the whole story.

In his papers, Lockwood expressly states that virulent streptococci definitely multiply faster in human serum containing peptone than in peptone-free human serum. Moreover, he declares and demonstrates graphically in 2 figures[1] that, following

[1] ref. (Lockwood, 1938a) figure 15, p. 183; (Lockwood, 1938b) chart, p. 805.

an inoculation of 3000 streptococci in peptone-free human serum, the organisms do not increase in number during the first 5 hours of incubation at 37.5°C. whereas after the same inoculum when peptone is present in the medium a marked increase in organisms occurs during this time (3000 organisms increase to approximately 600,000, see figure 2[2]). He also reports that human peptone-free serum is not a suitable medium for prolonged survival of hemolytic streptococci inasmuch as after 24 hours growth in this medium, progressive reduction of surviving organisms occurs. On the other hand, he says that human serum containing peptone supports the growth of the organisms for a much longer period of time. From these observations, is it not possible that the explanation, at least in part, of the greater effect of sulfanilamide on organisms growing in peptone-free human serum than on organisms growing in serum containing peptone, is merely that the organisms multiply more slowly in the former than in the latter medium and hence the sulfanilamide has fewer organisms, in the medium in which the peptone is excluded, on which to act and therefore has a correspondingly greater effect? In other words, sulfanilamide is effective under conditions unfavorable to rapid multiplication of the streptococcus and this condition is obtained when this organism is grown in peptone-free human serum. In regard to the preceding observation, all investigators appear to agree that in any one medium, all other conditions being alike, the effect of sulfanilamide is roughly inversely proportional to the number of organisms inoculated and that when relatively large inocula are used, even large concentrations of sulfanilamide cannot prevent the growth of the organism. Lockwood brings out this latter point himself when he says that with an initial inoculum of 250,000 per ml. even 0.1 mgm. per ml. of sulfanilamide in peptone-free human serum can merely maintain the initial population level.

As regards the effect of sulfanilamide on the growth of the streptococcus in rabbit serum at 37.5°C. with and without peptone, again we believe that the effect of sulfanilamide is, at least in part, directly correlated with the rate of multiplication of the

[2] ref. (Lockwood, 1938a) figure 15, p. 183.

organism. When the organisms multiply rapidly as they do in rabbit serum at 37.5°C. after a relatively large inoculum (3000 organisms) whether or not the serum contains peptone, sulfanilamide even in high concentration (1 mgm. per ml.) has only a slight restricting influence on the growth. When, however, a relatively small inoculum is used, (400 organisms per ml.) after which the organisms multiply relatively more rapidly in the medium containing peptone than in the peptone-free medium, sulfanilamide is bactericidal for the organisms growing in the peptone-free medium (40 hours) and merely bacteriostatic for those growing in the medium containing peptone.

Therefore, we believe that the explanation of the difference in the results obtained with sulfanilamide on organisms growing in human and in rabbit serum at 37.5°C. is mainly that rabbit serum, even when peptone is excluded from the serum, is a very good medium for the growth of the streptococcus, being able to support growth at 37.5°C. for at least 3 days, whereas human serum, expecially when peptone is excluded from the serum, is a poor medium for the growth of this bacterium. It is only when the growth of the organism in rabbit serum is retarded in some way, that the bactericidal properties of sulfanilamide at 37.5°C. become evident and can be compared with those occurring when human serum is used as a medium for growth.

That sulfanilamide when peptone is present is strikingly more effective when tests are carried out at 39 and 40°C. than at 37.5°C. was first observed by White and Parker and has now been confirmed by us. In regard to these marked bactericidal properties of sulfanilamide on the streptococcus multiplying at elevated temperatures, there appears to be at least one factor involved other than that of retardation of growth, since such retardation does not seem sufficient to explain the striking bactericidal effects encountered. We believe that such impressive bactericidal results are explicable only on the theory that sulfanilamide is more reactive at these elevated temperatures than at 37.5°C. The fact, noted previously, that sulfanilamide has only weak bacteriostatic qualities in tests run at 20°C. in spite of the slow growth of the organism at this low temperature appears to bear out this idea.

In view of the finding that streptococci always multiply with greater speed when peptone is present than when it is not, our results showing that at 39°C. sulfanilamide is less effective on organisms growing in peptone-free media than in media containing peptone is surprising. No explanation of this phenomenon can be offered at present.

CONCLUSIONS

We believe that these experiments dealing with the effect of sulfanilamide on the growth of the streptococcus in rabbit serum justify the following conclusions:

1. Sulfanilamide in moderate concentrations is bactericidal only for streptococci which are multiplying relatively slowly.
2. The bactericidal action of sulfanilamide appears to be increased when the organisms are grown at elevated temperatures
3. Our experiments do not seem to support the theory that sulfanilamide interferes with the power of streptococci to utilize serum proteins or that peptone *per se* interferes specifically with the bactericidal effectiveness of sulfanilamide.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

ILLINOIS BRANCH

CHICAGO WOMEN'S CLUB, CHICAGO, MAY 5, 1939

SIGNIFICANCE OF STREPTOCOCCI IN DENTAL CARIES. *Ruth Tunnicliff*, From the John McCormick Institute for Infectious Diseases and the Foundation for Dental Research of the Chicago College of Dental Surgery.

Smooth and microscopic rough colonies of *Streptococcus viridans* were isolated from 0.2 per cent glucose brain broth cultures of pulps of carious teeth and carious dentin. Bacillary, coiled forms, crescents and straight or undulating filaments in rough colonies were changed by transferring in 1 per cent glucose broth into cocci in pairs or short chains forming smooth colonies. Smooth colonies isolated from mouths with and without carious teeth may be changed into microscopic or minute rough colonies made up of the same morphologic forms found in colonies isolated as rough from carious dentin. Organisms in rough cultures produced less acid (pH 5.2–6.0) than those in smooth (pH 4.4–4.8) in 1 per cent glucose broth, pH 7.0. Organisms from both rough and smooth colonies grew in 1 per cent glucose broth, pH 4.4–5.0. Organisms found in smooth and rough colonies of *Streptococcus viridans* appear to correspond to the cocci, bacilli and "tortuous threads" described by Miller in tubules of carious teeth. Artificial caries showing these three forms in tubules was produced by dental strains of *Streptococcus viridans*. By weekly subculturing in 1 per cent glucose broth, pH 7, the cocci gave a pH 4.4 during the three months necessary to produce caries.

SOME STUDIES IN FOCAL INFECTION. *George W. Stuppy and Willard Wood*, Rush Medical College and the Presbyterian Hospital.

PUBLIC HEALTH EDUCATION AND THE NEWSPAPER. *Irvine S. Cutler*, Northwestern University Medical School.

CONNECTICUT VALLEY BRANCH

SMITH ALUMNAE BUILDING, SMITH COLLEGE, MAY 27, 1939

SOME EXPERIMENTS WITH STAPHYLOCOCCUS ENTEROTOXIN PRODUCTION. *Edwin L. Minard*, Department of Bacteriology, Yale University.

Attempts to titrate the staphylococcus enterotoxin by means of isolated intestinal strip of kittens were negative. These results tend to support the hypothesis that the enterotoxin acts primarily through the nervous system. A further method for the titration of enterotoxin by the floccu-

lation procedure, using antiserum prepared in the rabbit, was unsuccessful, except with the antiserum of one of a large series of rabbits. A specific flocculation occurred when the antiserum of this rabbit was mixed with a filtrate containing only active enterotoxin.

Gelatin hydrolysate medium amplified with the growth accessory factors, nicotinic acid and thiamin, together with the amino acids, tryptophane, tyrosine and cystine, was found to be almost as satisfactory a medium for enterotoxin production as the complex peptone medium employed. The addition of agar to give a semisolid consistency greatly increased the enterotoxin titer of the filtrate.

SEROLOGICAL TYPES OF STREPTOCOCCUS UBERIS. *W. N. Plastridge and L. F. Williams*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Observations were made on the serological properties of 141 cultures identified by biochemical tests as *Streptococcus uberis* (Diernhofer) which is apparently identical with group III of the English workers, and group Ba (Storrs).

The cultures used produced either weakly beta hemolytic or non-hemolytic colonies on blood agar, split aesculin (Edwards' medium), acidified litmus milk with slight or no reduction, produced variable results in methylene blue milk (1–5000), usually hydrolyzed sodium hippurate, fermented lactose, mannitol, sorbitol, trehalose and usually inulin, and failed to ferment raffinose and arabinose. Of 141 cultures examined by the slide agglutination test, 136 were placed in 11 serological types and 5 failed to react with any of the type sera employed. While the majority of the cultures were type-specific, about 20 per cent shared a common antigen with more than one serological type.

On the basis of biochemical characteristics, it appears that *S. uberis* does not belong in the *Viridans*, *Lactic* and *Enterococcus* divisions of Sherman (1937). His *Pyogenic* division consists of Lancefield's serological groups A, B, C, E, F, G and H. Biochemically, *S. uberis* resembles Lancefield's group E more closely than any other group. A minor (group ?) antigenic relationship was observed between our serological type 2 and Lancefield's group E. None of the *S. uberis* cultures were agglutinated by antisera against groups other than group E.

IMMUNITY IN RESPONSE TO VACCINATION WITH SPECIES OF THE PROTEUS GENUS. *Walter L. Kulp*, Laboratory of Bacteriology, Connecticut State College, Storrs, Connecticut.

Living and heat-killed cultures of *Proteus mirabilis* and *Proteus vulgaris*, used as vaccines, produced a high degree of immunity in mice against *Bacillus anthracis*, a hemolytic streptococcus of human origin and *Proteus hydrophilus*. Against the pneumococcus, this vaccination procedure manifested itself only in lengthening the period before death to approximately four times that period in controls. These immunizing procedures induced no protection against *Salmonella typhimurium*. Egg white, milk and blood serum as vaccines did not initiate any protective effect against *P. hydrophilus*.

Filtrates from *Proteus hydrophilus* proteose-peptone broth cultures of varying ages caused only slight toxic effects in mice when amounts up to

1.0 ml. were injected intraperitoneally. Subsequent tests indicated that these filtrate-injected animals were immune to lethal doses of *P. hydrophilus*. Oral administration of living *P. hydrophilus* over an extended period had no immunizing effect. In practically all cases, no antigenic relationship between the vaccine material and the pathogen employed could be demonstrated. Work is in progress in an attempt to determine duration of immunity and the factors involved.

Bactericidal Properties of Allyl Isothiocyanate and Some Related Oils. *Milton J. Foter*, Department of Bacteriology, Connecticut State College, Storrs, Connecticut.

In an attempt to attribute the inhibitory property of the vapors from crushed horse-radish to some definite compound or compounds, a study was made of Allyl Isothiocyanate the main volatile constituent of horse-radish and several oils similar in structure, namely Methyl Isothiocyanate and Ethyl Isothiocyanate.

The inhibitory properties of the vapors from the oils and the effect of various dilutions of the oils directly were studied by three different methods using ten test organisms. The results indicate that Allyl Isothiocyanate is apparently responsible for the inhibitory effects of the vapors from crushed horse-radish on microorganisms and that Methyl and Ethyl Isothiocyanate likewise possess strong bactericidal properties.

A Comparative Study of Toxic Extracts of the Enteric Fever Group. *Eleanor Hague*, Department of Hygiene and Public Health, Smith College, Northampton, Mass.

Extracts were prepared from smooth and rough cultures of *Eberthella typhi*, *Salmonella enteritidis*, and *Salmonella aertrycke*, by heating washed, alkaline, ice water suspensions of the organisms four hours at 60°C., centrifuging, and filtering the supernatant liquid through Berkefeld or Seitz filters.

The minimum lethal dose was taken as the least amount which killed three out of five mice in 24 hours. For the extracts of the smooth strains of organisms this dose ranged from 0.3 ml. to 1 ml., while the dose for extracts from rough strains was from 1 ml. to 3.5 ml. The extract from a smooth culture was always at least twice, if not three times, as toxic as the extract of its corresponding rough strain.

Antiserums were produced by injecting rabbits intravenously with small graded doses of the sterile filtrates. The smooth bacterial extracts were comparatively antigenic while two extracts from rough strains of organisms were just slightly antigenic.

The protective potency of the immune serums against the extracts was tested by injecting a group of 5 mice intraperitoneally with 0.5 ml. of antiserum and one-half hour later $1\frac{1}{2}$ or 2 minimum lethal doses of extract. The amount of protection was recorded in average number of hours before death for the group. The extracts from rough strains of organisms, although less toxic, were affected less by homologous or heterologous antiserums from smooth and rough bacterial extracts than were the extracts from smooth organisms. There was little apparent difference between the protective power of smooth and rough bacterial extract antiserums against the rough bacterial extracts. Controls with normal serum afforded as good protection as any of the immune serums against

the rough bacterial extracts. The protection afforded against smooth bacterial extracts showed a definite trend. All immune serums afforded better protection than normal serum, and that against common O antigenic fractions better than that against unrelated heterologous extracts. The protective potency of rough bacterial extract antiserums demonstrated against smooth bacterial extracts may be due to smooth factors present in the rough strains from which the extracts were produced.

ERRATUM

In the abstract of a paper on "The activity against type-VIII pneumococcus of an enzyme produced by a soil microorganism grown on type-VIII polysaccharide," by Grace M. Sickles and Myrtle Shaw, J. Bact., August, 1939, *38*, 241–242, the generic name of the organism should be *Bacillus* and not *Rhodobacillus*.

PULLULOMYXA BOTRYTIS N. SP.

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A brief note was published some years ago by W. T. Morgan of the Lister Institute, London, and the writer (1932) describing the isolation from decayed woody tissues of a micro-organism which decomposes the specific polysaccharides of *Shigella dysenteriae*, Shiga; *Shigella dysenteriae*, Flexner Y; *Pneumococcus* Type II; and the tubercle bacillus.

At the time a study of the relationship of this organism was not proceeded with as it was felt that its morphological peculiarities deserved closer attention than it was then possible to devote to them. The organism was provisionally referred to as a *Myxococcus*, a designation which was based solely on its microscopic appearance. Strained *in vivo* with a dilute solution of methylene blue it resembles the coccoid stage of many myxococci as depicted by Krzemieniewscy (1928), and incidentally of *Spirochaeta cytophaga* as described by Hutchinson and Clayton (1919), an organism which Kremienewska (1930) states approaches the myxococci in its characters.

Figure 1 shows the polysaccharide-decomposing organism stained *in vivo* with dilute methylene blue. Its apparently thick-walled coccoid cells contain deeply staining plasmatic inclusions which, in many cases, appear to adhere to one side of the cell wall. The cells measure between 2.5 and 3 μ in diameter; a few may exceed 3 μ and some, not including those attached to larger cells, may be no more than 2 μ in diameter. In the production of smaller coccoid cells by normally sized spheres the organism differs strikingly from the coccoid stage of the myxococci. These attached smaller coccoid cells can be seen to be

the daughter cells of the normally sized spheres, *arising from the latter by a process of budding*. There is therefore only one cell form in the life cycle of the polysaccharide-splitting organism.

Among the myxococci, on the other hand, the coccoid stage represents the resting stage in the life cycle: on germination it gives rise to rod-shaped cells. These rods are the active or reproductive stage of the life cycle. Only when reproduction ceases does a coccoid stage again appear among the myxococci

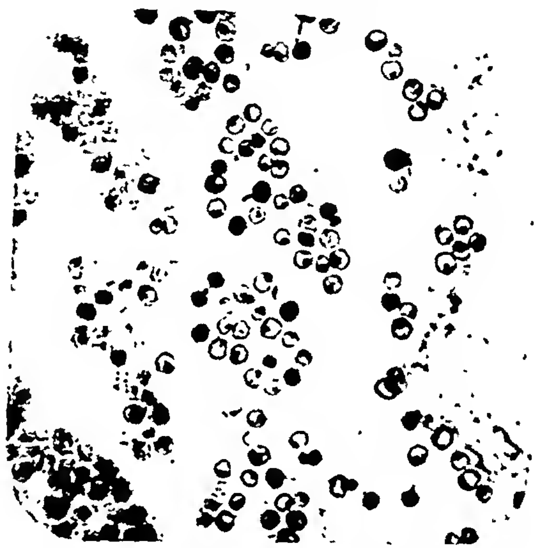

FIG. 1. THREE-DAY-OLD CULTURE OF *PULLULOMYXA BOTRYTIS*, STAINED IN VIVO WITH METHYLENE BLUE. × 2550 APPROX.

and this through the contraction of rodshaped cells into spheres, never as a result of the *budding of coccoids*.

It is true that in other groups of myxobacteria the two stages in the life cycle are morphologically more uniform than is the case among the myxococci. But in these cases the cell form is invariably rod shaped, both during the resting stage and the reproductive stage. No case has so far been described in which an organism belonging to the *Myxobacteriales* has shown a complete absence of rod-formed cells and a mode of reproduction reminiscent of budding.

It is for this reason that the writer has felt compelled to refrain from incorporating the organism here described among the *Myxobacteriales*.

The observation that reproduction in the polysaccharide-splitting organism takes place by budding naturally led to a comparison of this process as it occurs here with the budding process among the yeasts in order to see whether the new organism could be incorporated among the latter. But here again the writer was unable to find convincing evidence for doing so. In

FIG. 2. TWENTY-FOUR-HOUR CULTURE OF SACCHAROMYCES ELIPSOIDEUS, STAINED WITH FEULGEN'S STAIN. × 950

fact the observations which he made led him to conclude that very considerable differences exist between the process of budding in the two cases, notably in the behaviour of the thymonucleic substances.

Among the yeasts, reproduction by budding proceeds, as shown in figure 2, by the formation of a daughter cell which invariably attains a considerable size before part of the thymonucleic substances of the mother cell proceeds towards it, and eventually enters it through the narrow connection between the two cells.

The process of transfer of thymonucleic substances from the mother cell to the daughter cell is comparatively simple. The original spherical form of this material will stretch to produce a shorter or longer thread depending on the distance it is removed from the connecting passage between mother and daughter cell. Part of this thread will squeeze through the passage and form a sphere in the daughter cell, whilst the remainder, still in the mother cell, will again contract and form a sphere. The partition of the thymonucleic substances in the yeast cell thus corresponds to an amitotic nuclear division. The staining method adopted to

FIG. 3 FIG. 4

FIG. 3. SEVENTY-DAY-OLD CULTURE OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 2550 APPROX.
FIG. 4. FORTY-EIGHT-HOUR-OLD CULTURE OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 4000 APPROX.

ascertain the behaviour of the thymonucleic substances was based on that of Feulgen (1926).

The behaviour of the thymonucleic substances during the cell division of all the other yeast types which the writer has been able to examine, including a species of the small anascosporogene yeast *Pityrosporum rhinoscrosum*, was exactly the same as that described above for a typical *Saccharomyces ellipsoideus*.

In the polysaccharide-splitting organism here discussed, the behaviour of the thymonucleic substances during budding is more complex. In the resting cell this material forms a well defined sphere usually in the centre of the cell, as shown in figure 3, illustrating a 70-day-old culture stained with Feulgen's stain. At this age the cells are smaller than those of a young and active

culture. When growth commences, and before any extraneous signs of budding are noticeable, the sphere of thymonucleic substances elongates to form an equatorial band in the cell. Eventually this band may stretch further and become U shaped. In other cases the band of thymonucleic substances is represented by two parallel placed rods. These early stages can be observed in figures 4, 5, 6 and 7.

It has not been possible to ascertain with certainty the details of the further subdivision of the thymonucleic substances as revealed by Feulgen's stain. A closer scrutiny of figures 4 and 5 will show cells with two and three separate globules of these substances, indicating perhaps a further subdivision of the two above-described parallel rods, or possibly of the original U shaped

FIG. 5 FIG. 6 FIG. 7

FIGS. 5, 6 AND 7. FIVE-DAY-OLD CULTURES OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 4000 APPROX.

band. In some cases a subdivision of the substances into 4 separate units has been observed, and it cannot be excluded that the stage of three or of two separate globules may represent a subsequent fusion of a previously larger number of units. Throughout the period of subdivision of the thymonucleic substances the cell has remained globular without excrescences. However, when the stage of the existence of two or three definite globules of thymonucleic substances has been reached the cell can be seen to acquire a slightly egg-shaped form with one granule of thymonucleic substances eventually occupying the pointed end of the cell. Here the bud finally arises.

The mode of separation of the daughter cell from the mother cell in the polysaccharide-splitting organism also differs from that

of the yeasts, or at any rate from that of the genus *Saccharomyces* in which, according to Guilliermond (1920) and Lindner (1930), a transverse cellwall is formed between mother and daughter cell prior to the separation of the two cells. In the polysaccharide-decomposing organism on the other hand the two cells draw away from each other leaving a connecting filament between them which eventually breaks (see fig. 1), and may remain attached to one or both cells for some time as a short stalk. Where the daughter cell has produced a daughter cell prior to separation from its mother cell a short chain of three individuals may be formed. Such structures are strikingly similar to those described by Baker (1933) under the name of "giant cocci." Baker found such giant cocci in large numbers in cavities of the cellulosic content of the intestine of the guinea pig, where he associated them with the breakdown of hemicelluloses and possibly of cellulose.

The data recorded in the previous pages have convinced the writer that the polysaccharide-decomposing organism differs fundamentally from the yeasts and that, in fact, it is impossible on the available information to group it with any existing type of microorganism, including that of the myxococci to which, as already mentioned, early investigation had indicated that it might belong. Under the circumstances, he has thought it desirable to regard it as a new species, the name of which should indicate the most characteristic property of the organism, that of reproduction by budding, and to leave it to future investigations to decide its true position among the known groups of microorganisms, if it should be found of sufficient interest to deserve future attention.

At the suggestion of Dr. Ramsbottom of the British Museum the writer proposes that the organism be known as *Pullulomyxa botrytis* n. sp.

For the further characterisation of *Pullulomyxa botrytis* the following data have been compiled.

Motility. The organism is non-motile.

Staining properties. The ordinary aniline dyes are readily absorbed. A young culture of 2 to 3 days growth is gram posi-

tive, the stain giving good pictures of the cellular contents and of the connecting filaments between adjacent, directly related cells. A 20-day-old culture is still essentially gram positive though some of the cells will be found to take the counter stain.

Growth on agar media. No growth can be obtained on ordinary standard agar media. Even on the specially devised agar medium, growth is not visible during the first one or two days at the optimum temperature.

After 6 days incubation at 30 to 37°C. the surface colonies have attained a size of from 50 to 100 μ with an average of 80 μ. Both surface and embedded colonies are circular, with a smooth edge and a slightly granular interior. They are greyish white, transparent and moist. It is noticeable that the size of the colonies is larger on plates with a larger number of colonies than on those containing a few only. On older plates this difference is reversed and after 14 days the sparsely seeded plates may show colonies of a diameter of from 0.4 to 1.2 mm., while those on thickly seeded plates fail to exceed 0.4 mm. Older colonies are no longer transparent but retain the original moist appearance. Their colour is no longer greyish white, but faintly brownish grey.

Growth on gelatin. Growth is not easily obtained since the temperature of incubation is well below the optimum of the organism. After 17 days incubation no growth could be observed on the inoculated plates. Nevertheless, gelatin is very slowly liquefied by the organism. This was shown by adding a thick suspension of living cells to a tube of ordinary gelatin and incubating the latter at 37°C. for 17 days. By then the gelatin, when cooled, could not be made to solidify though control tubes without an addition of the organism did so.

Growth in liquid media. In the most favourable medium, described subsequently, visible growth may be noticeable at 37°C. within 24 hours. The clear liquid has become very faintly turbid and slightly bleached. Turbidity and bleaching increase slowly during the subsequent period of incubation until, after 8 days at 37°C., the medium is markedly turbid with a noticeable greyish sediment. When growth in liquid media takes place in shallow layers it is more rapid, and marked turbidity and sedi-

mentation is already noticeable after 24 hours incubation at 37°C. The more rapid development causes the cells to accumulate in clusters which may be sufficiently large within the first 48 hours of growth to be visible with a hand lens. The rapid growth in shallow layers confirms the aerobic nature of the organism. Even with the addition of suitable carbohydrates, growth is not possible under strictly anaerobic conditions.

Temperature range of growth. Initial growth, during the first 24 hours of incubation is very similar in extent throughout the range tested, between 18 and 40°C. Subsequently, however, little progress in growth is made in cultures kept at the former temperature and even at 25°C. The range from 30 to 37°C. appears to be suitable for a normal growth rate, and there is little to choose between the two extremes. The higher temperature of 40°C. has not been found suitable for the maintenance of cultures.

Lethal temperatures. A young culture kept for 30 minutes at 52°C. was found capable of propagating when subcultured into fresh medium and incubated at 37°C. An identical culture kept for the same period at 55°C. failed to do so.

pH requirements of the organism. The most abundant growth is observed when the standard medium is adjusted to a hydrogen-ion-concentration range of between pH 7.0 and 7.6. This growth can be maintained even at pH 8.0, but no growth was noticeable at a pH of 8.6. On the acid side of the neutral point, a certain amount of growth could be induced in the standard medium at a pH of 6.5 but little if any at 6.0 and below.

Composition of standard medium. When the organism was first discovered it was seen under the microscope in a medium composed of ammonium sulphate 0.01 per cent, dipotassium hydrogen phosphate 0.02 per cent and water. To this had been added 0.001 per cent of the specific polysaccharide of *Shigella dysenteriae*, Shiga. The rate of growth of the organism was very slow in this medium and it was found that the addition of a certain amount of an extract made from fresh rabbits' droppings greatly increased the rate of growth and facilitated the eventual isolation of the organism.

All subsequent work, and the cultivation of *Pullulomyxa*

botrytis, has been done in a medium the composition of which is based on the above observation.

The following procedure is adopted in the preparation of the standard medium: 10 gr. of fresh rabbit pellets are left over night at room temperature in 1 litre of tap water. The extract obtained is poured off, and rejected. It is replaced by a further litre of tap water, with which the pellets are given a short boil. The second extract thus obtained is filtered and to the clear liquid are added 2 g. of ammonium sulphate, 4 g. of dipotassium hydrogen phosphate, and 4 g. of a carbohydrate such as fructose or xylose. The volume of extract is finally made up to 2 litres and sterilised. When solid media are required, 1.5 per cent of agar agar are dissolved in the standard medium prior to its being sterilised. The sterilisation has usually been done fractionally on three successive days with one hour's steaming daily.

On or in the above medium, *Pullulomyxa botrytis* grows more abundantly than on any other tried. This, however, is not meant to imply that the growth of the organism, even under the most favourable conditions, is really copious, but merely that it is markedly richer than on the ordinary bacteriological culture media, which in most cases are unsuitable.

Utilisation of carbohydrates. In the preliminary note which appeared in Nature (1933) it was mentioned that *Pullulomyxa botrytis* destroyed the specific properties of a number of bacterial polysaccharides. This was thought to indicate that the saccharolytic enzyme complex of the organism would be able to function on a wide range of carbohydrates, since it is doubtful whether the organism could have met with polysaccharides of pathogenic bacteria in its natural habitats. It was thought of interest therefore, to test the action of *Pullulomyxa botrytis* on a wide range of carbohydrates, including several polysaccharides.

Of these, cellulose was found to remain unaffected. Gum arabic, however, and xylan as well as a polysaccharide isolated by Campbell (1935) from oak sapwood were suitable sources of carbohydrate. Starch and inulin did not promote growth. Among the disaccharides tested, cellobiose, maltose, sucrose and lactose could be utilised, the latter less readily than the three

former. Of the monosaccharides, fructose appeared more suitable than glucose. In fact it is questionable whether glucose can readily be utilised. The two pentoses tested, xylose and arabinose, were both suitable. Growth for prolonged periods on any of these carbohydrates did not in the least weaken the action of the organism on the specific bacterial polysaccharides which it was originally found capable of destroying.

It is of interest to note that *Pullulomyxa botrytis* is capable of utilising not only hemicelluloses which occur widespread in nature, but also an intermediate product of hydrolysis of cellulose. It will be remembered that attention was drawn above to the similarity in morphology between this organism and the "giant cocci" observed by Baker (1933) in the coecum of certain herbiverous animals. From a physiological point of view, therefore, a similarity also exists between the two types.

The action of *Pullulomyxa botrytis* on the various carbohydrates mentioned as suitable for growth did not lead to the production of visible quantities of acid or gas. It was decided, therefore, to study this action in the Barcroft manometer, and to determine the oxygen uptake of an active suspension of the organism on some of the carbohydrates which had been previously tested for their suitability as energy suppliers.

Using a suspension in saline of a 10-day old culture grown on xylose agar, xylose showed an oxygen uptake of 28 μl within 4 hours and a gas evolution of 52 μl, assuming this gas to have been carbon dioxide. On the assumption of the evolved gas being hydrogen the oxygen uptake was 80 μl within the first 4 hours and the gas evolution 52 μl as before.

The same suspension of the organism gave with fructose an oxygen uptake of 40 μl assuming the gas evolved during the reaction to have been hydrogen. In the case of this particular carbohydrate no carbon dioxide can have been evolved.

Glucose was unable to show an oxygen uptake by the suspension, even when the experiment was continued for 24 hours.

A considerable amount of work was devoted to the isolation of the enzyme system of *Pullulomyxa botrytis* which causes the destruction of the polysaccharides of bacterial toxines. These

efforts, however, were completely unsuccessful. Neither by autolysis, nor by freezing and disintegration of the living cells could the responsible enzymes be separated in active form. It has not been possible, therefore, to study the effect on infected animals of an injection of the polysaccharide-splitting enzyme of *Pullulomyxa botrytis.*

CONCLUSIONS

A description is given of the cytological, morphological and physiological characters of an organism which was isolated from decaying vegetable debris and which previous work had shown was capable of destroying the specific polysaccharides of certain bacterial toxins.

The cytological study has shown that the organism in question, for which the name *Pullulomyxa botrytis n. sp.* is suggested, represents a type which, to the writer's knowledge, has never before been described in the literature.

Its propagation proceeds by budding, but the cytological changes involved are far more complex than those met with in the yeasts.

Details are given of the morphological and cultural characteristics of *Pullulomyxa botrytis* n. sp. and a reference is made to the work which has been done on the isolation of the enzyme system by which the specific polysaccharide of the Shiga dysentry toxin must be assumed to be destroyed by the organism.

The writer wishes to place on record his thanks to Dr. Ramsbottom of the British Museum for his advice on nomenclature; to Mr. W. G. Campbell of the Forest Products Research Laboratory of the Department of Scientific and Industrial Research, for the supply of a sample of oak softwood polysaccharide; and to Mr. K. R. Butlin of his staff, for the determination of the respiratory properties of *Pullulomyxa botrytis.*

The work described above was carried out as part of the programme of the Chemistry Research Board, and is published by permission of the Department of Scientific and Industrial Research, London, England.

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STUDIES ON CAPSULE FORMATION

I. The Conditions under Which Klebsiella pneumoniae (Friedländer's Bacterium) Forms Capsules

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INTRODUCTION

Several genera of bacteria are characterized by the possession of slimy envelopes, usually called capsules. The presence of such capsules may be demonstrated microscopically by certain staining methods or by the India Ink method of Burri (1909). Capsules are particularly well marked in certain species, notably the *Pneumococcus* group, the *Klebsiella* group, the *Salmonella* group, *Bacillus anthracis*, *Clostridium welchii* and *Gaffkya tetragena*. There are serological indications, however, that many more species of pathogens possess envelopes, very similar to capsules, although these envelopes do not have the extreme size which makes them readily demonstrable microscopically.

A close relationship appears to exist between encapsulation and pathogenicity. It need not be stressed, therefore, that a better understanding of the conditions under which certain species form capsules might be of great value in regard to problems concerning the pathogenicity of micro-organisms. Little, if any, work has been done in this field, most of the attention having been concentrated either on the physico-chemical nature of the capsular substances, or on the serological problems related to encapsulation.

To define the conditions under which one species of an encapsulated pathogen (Friedländer's bacterium) forms its capsules has been the aim of this investigation.

METHODS

In preliminary work we attempted to apply the method of Burri to the comparison of the sizes of the capsules of bacteria derived from different cultures. The results were disappointing, the method depending too much on personal judgment, and being influenced greatly by slight irregularities in technique. Only marked differences in capsule size could be demonstrated. This method, therefore, was abandoned.

Growing cultures of Friedländer's bacterium become very viscous when abundant capsule formation takes place, but practically no change in viscosity can be observed when the organism does not form capsules. With this fact in mind the possibility was considered whether measuring the relative viscosity would not provide a more accurate method of comparing average capsule sizes. This, indeed, appeared to be possible.

From a theoretical point of view, one may expect a direct proportionality between relative viscosity and total volume of the suspended particles (in this case the total volume of the encapsulated bacteria) according to the equations of Einstein (1911), von Smoluchowski (1916), and Jeffery (1922). Such equations, from which can be calculated mathematically the size of the capsules, hardly can be expected to be valid in a system so complex as a culture of encapsulated bacteria. Nevertheless, there was evidence that the larger the capsules, the higher the relative viscosity.

Since, however, the relative viscosity of a growing culture is dependent not only on the size of the capsule but also on the number of encapsulated bacteria, a comparison is possible only when the same number of encapsulated bacteria are present. It is necessary, therefore, to make bacterial counts and to plot the relative viscosity observed, against the number of bacteria responsible for this viscosity. From the position of such curves definite conclusions can be drawn as to differences in the average size of the capsules of bacteria derived from different cultures. By using this technique it was possible to demonstrate small differences in average capsule size, which could not, with sufficient accuracy, be demonstrated by the Burri method.

Relative viscosity was measured in the ordinary Ostwald capillary viscosity tubes at 37°C. Since this kind of viscosimeter may be sterilized and handled under sterile conditions, no alterations for bacteriological purposes are necessary. Furthermore, with a single growing culture, as many readings as desired may be made without endangering the sterility of, or sacrificing the culture.

Sterile viscosity tubes were filled with 5 ml. of nutrient broth, previously inoculated with a vigorously growing culture of Friedländer's bacterium and viscosity measurements made at half-hourly intervals after the initial inoculation. Bacterial counts were made using the Levy-Hausser counting chamber and a suitable dilution of the culture.

EXPERIMENTAL

The strain of Friedländer's bacterium used throughout this work was obtained from the American Type Culture Collection (*Klebsiella pneumoniae* No. 4727). The strain was originally isolated and described by Schlossberger and Menk (1930). Several animal passages of the strain were made before this work was begun. Large slimy colonies were formed on solid agar, and in liquid medium extremely high viscosities were obtained under special conditions. An example of such an increase in viscosity during the growth of Friedländer's bacterium, in 4 per cent neopeptone-1 per cent glucose, is shown in figure 1-a.

A few hours after incubation the viscosity begins to increase, until, after 7 hours the relative viscosity has attained a value of almost 6. In this state, large capsules may be demonstrated by the Burri method, and the culture has a slimy consistency which may be seen readily when an inoculating needle is thrust into the tube and withdrawn with a long slimy thread adhering.

It is readily demonstrated that the high viscosity in full-grown cultures of Friedländer's bacterium is due to the presence of intact capsules and is not due to excretion of capsular substance in the medium. It appeared to be possible to disrupt the capsule from the bacteria by a purely mechanical method and to bring the capsular substance into solution without destroying the

bacteria themselves to any appreciable extent. This was done by exposure for about three minutes to sonic vibration in a sonic oscillator wherein a nickel rod is brought into rapid vibration by an alternating magnetic field. The fluid, contained in a glass cylinder surrounding this nickel rod, is also submitted to these

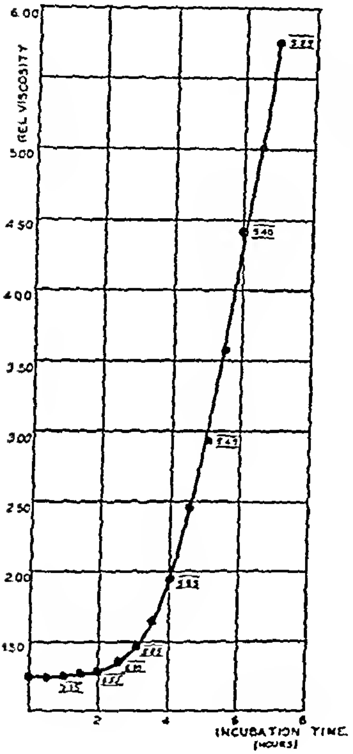

FIG. 1-a

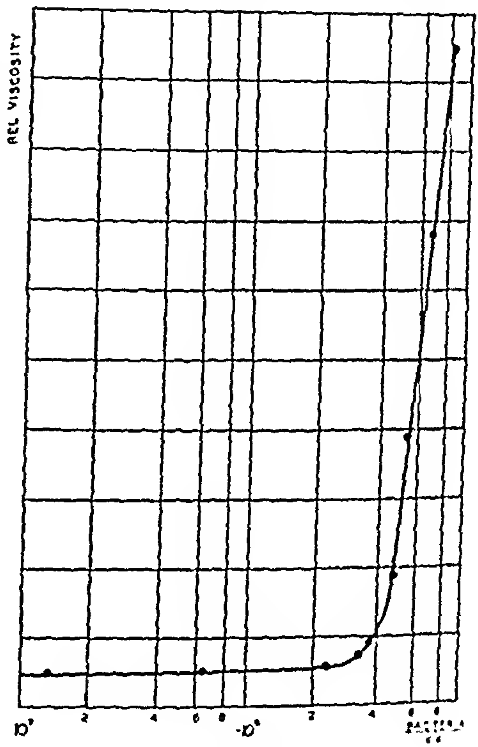

FIG. 1-b

FIG. 1-a. Increase in relative viscosity of a culture of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose, during 7 hours incubation at 37°C. (initial pH 7.5) (pH changes during growth are shown).

1-b. Relative viscosities of the same culture shown in figure 1-a in relation to the number of bacteria present at varying times during incubation.

vibrations and it appears that encapsulated bacteria present in this fluid quickly lose their capsules.

When a very viscous, full-grown culture of Friedländer's bacterium was submitted to such treatment, it was found that in a very short time the capsules disappeared (Burri method).

At the same time an enormous drop in the viscosity of the culture to almost that of the original broth was observed. Figure 2 shows the result of sonic vibration treatment on the viscosity of a young culture of Friedländer's bacterium in neopeptone-glucose. Dilution series were made from this culture before and after treatment, and also after part of the culture had been heated for 10 minutes in a boiling waterbath. For each dilution, in which the numbers of bacteria were known, relative viscosity was measured. As may be seen from figure 2, high viscosities were observed only

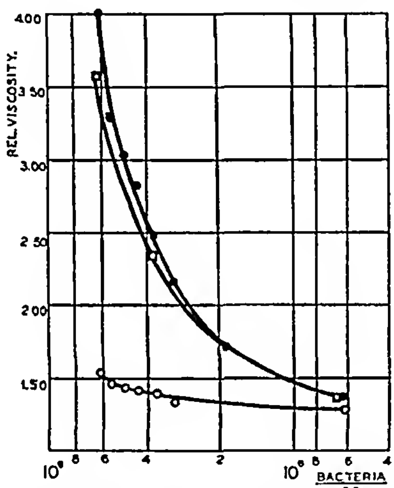

FIG. 2. Relative viscosities of a full grown culture of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose and different dilutions of this culture with the original nutrient medium. ●——● untreated. □——□ same culture, 10 minutes boiled at pH 8.8. ○——○ same culture after 2 minutes sonic vibration.

when the structure of the capsule was maintained. The same amount of capsular substance, disrupted from the bacterial cells and dissolved, caused hardly any increase in viscosity. Boiling for 10 minutes did not destroy the capsule, either when the medium was slightly alkaline (pH 8.8), or when it was slightly acid (pH 4.4). A direct correlation therefore appeared to exist between the relative viscosity and the presence of capsules on the bacteria. Figure 2 also gives an example of the interrelation of the relative viscosity and the concentration of encapsulated

bacteria. From a great number of such dilution curves of very viscous cultures it was found that the following equation could be applied:

$$\log \cdot \frac{\eta_s - \eta_0}{C\eta_0} = a + bC$$

In this empirical equation C represents the number of encapsulated bacteria present, $\frac{\eta_s - \eta_0}{\eta_0}$, the *relative viscosity for this* number of bacteria, and a and b are constants. This formula is identical with the one found to be valid for the interrelation of the relative viscosity and sol concentration of many hydrophilic colloids (Bungenberg de Jong, Kruyt and Lens 1932).

It appears, however, that this equation cannot be applied to the interrelation between relative viscosity and number of bacteria during growth (fig. 1-b). A closer study of figures 1-b and 2 reveals that the curves are decidedly different, thus indicating that the capsule size is not the same during the entire growth period. This was an unexpected result and so was studied more fully. At regular intervals, counts and viscosity measurements were made on a growing culture of Friedländer's bacterium. A curve similar to the one represented in figure 1-b was thereby obtained. In addition, at 3, 4½, 6 and 7½ hour intervals after inoculation, dilution series were made with the same kind of broth in which growth occurred, and viscosities of these dilutions estimated. The results of these experiments are shown in figure 3.

Figure 3 indeed shows that the capsular size in a growing culture of Friedländer's bacterium is not constant. The dilution curves do not coincide as might be expected if capsular size were constant over the whole range of growth. In the logarithmic phase of growth encapsulation is very poor. The capsule size increases rapidly in the later stages of growth.

By measuring the pH during growth, it appeared that encapsulation occurs mainly when the pH is as low as 5.6 to 5.0, therefore, under conditions unfavorable for further multiplication. Such conditions are reached 4 hours after inoculation. After

6 hours, the capsules have attained their optimal size and do not increase further. Seven and one-half hours after inoculation, capsules are the same size as at 6 hours (both dilution curves practically coincide).

The fact that capsules are formed mainly after the logarithmic period of growth was demonstrated clearly, not only with the

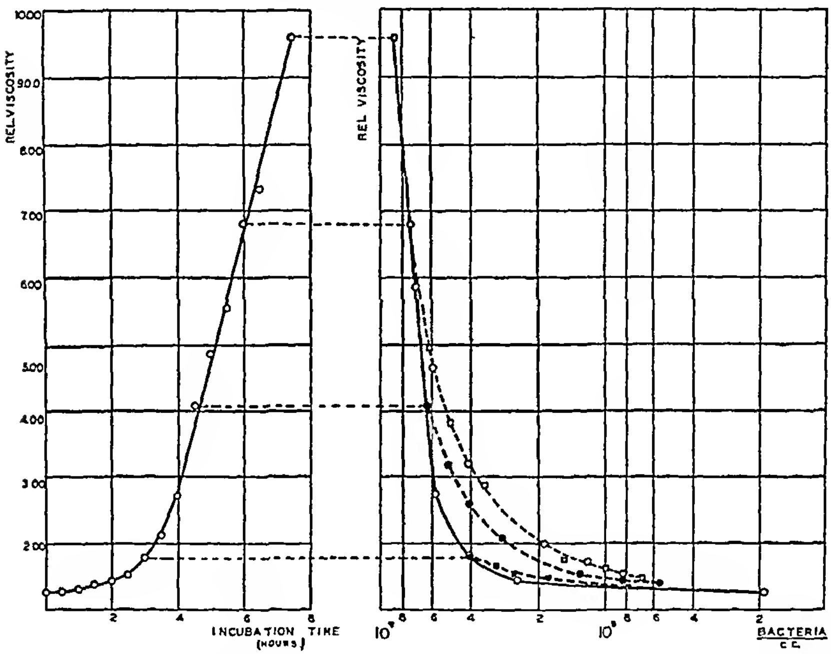

FIG. 3-a FIG. 3-b

FIG. 3-a. Increase in relative viscosity of a culture of Friedlander's bacterium in 4 per cent neopeptone-1 per cent glucose during 7½ hrs. incubation at 37°C. (initial pH 7.5).

3-b. Relative viscosities of the same culture in relation to the number of bacteria present at varying times during incubation (solid line). Viscosities of dilutions of the same culture made with the original nutrient broth at 3, 4½, 6 and 7½ hours incubation (broken lines).

relative viscosity technique, but also with India ink stains, taken at hourly intervals after inoculation (plates 2 and 3).

INFLUENCE OF SUGAR ON CAPSULE FORMATION

Since the capsular substance of Friedländer's bacterium is carbohydrate in nature it seemed of interest to determine whether

the glucose in the culture medium was a factor contributing to the formation of capsules.

It appears that the presence of carbohydrate is not strictly necessary for capsule formation. When cultivated in a 4 per

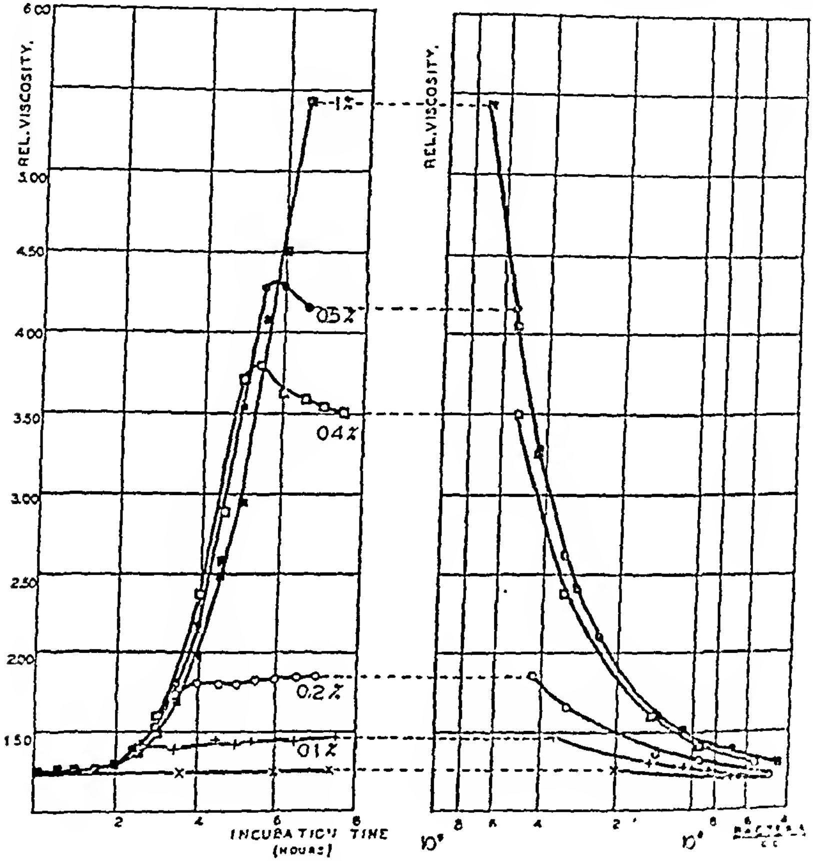

Fig. 4-a Fig. 4-b

Fig. 4-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone with addition of different amounts of glucose varying from 0 to 1.0 per cent at 37°C. (initial pH 7.5).

4-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

cent neopeptone solution at pH 7.5 as well as at pH 5.8, the presence of small capsules could be demonstrated by the India ink method after 6 hours of incubation (Plate 1). However, the capsules formed in sugar-free media are far inferior in size to

those obtained in the same medium with added sugar (Plate 3). It also appears that glucose, although not strictly necessary for the formation of capsular substance, plays an important rôle as a component of capsular substance. Figure 4 shows that practically no increase in viscosity is observed when glucose is eliminated from the medium, while the glucose-containing controls show a marked increase. However, this difference is due in part to the very restricted growth in the glucose-free culture, (2.0×10^8 bacteria per ml. compared with 7.0×10^8 bacteria per ml. in the control) at the end of the experiment.

When small amounts of glucose are added (0.1 to 0.5 per cent) (fig. 4) growth and capsule formation occur normally until all of the sugar present has been utilized. If no capsules have been formed during this period, they are not formed thereafter. At least 0.3 per cent glucose is necessary for the production of well-encapsulated bacteria. When all of the sugar has been fermented, multiplication continues, but bacteria developing under these (sugar-free) conditions are very poorly encapsulated. Therefore, a marked drop in viscosity is observed as soon as all of the glucose has disappeared, notwithstanding a slight increase in the bacterial count. The capsule size decreases as soon as the glucose supply is exhausted. Addition of formalin, at the moment when all of the sugar has disappeared, stops further proliferation and therefore no drop in viscosity is observed after further incubation, the capsule size remaining unchanged.

Higher glucose concentrations (up to 10 per cent) very markedly inhibit capsule formation. The greatest capsule size is obtained by adding from $\frac{1}{2}$ to 1 per cent glucose to the culture medium. Lower concentrations are insufficient to obtain the conditions favorable for capsule formation, while higher concentrations are inhibitory.

The utilization of several other fermentable substrates in the synthesis of the capsular polysaccharide was studied. One per cent solutions of different sugars and other fermentable substrates in 4 per cent neopeptone broth were inoculated with Friedländer's bacterium, incubated, and each half hour the relative viscosity

was measured. Figure 5 shows the results of these experiments. As may be seen the most rapid increase in viscosity occurs in the presence of glucose, but considerable increase occurs also in the presence of sucrose, maltose and mannitol. Pyruvic acid, glycerol, rhamnose and salicin give less marked increases. Lac-

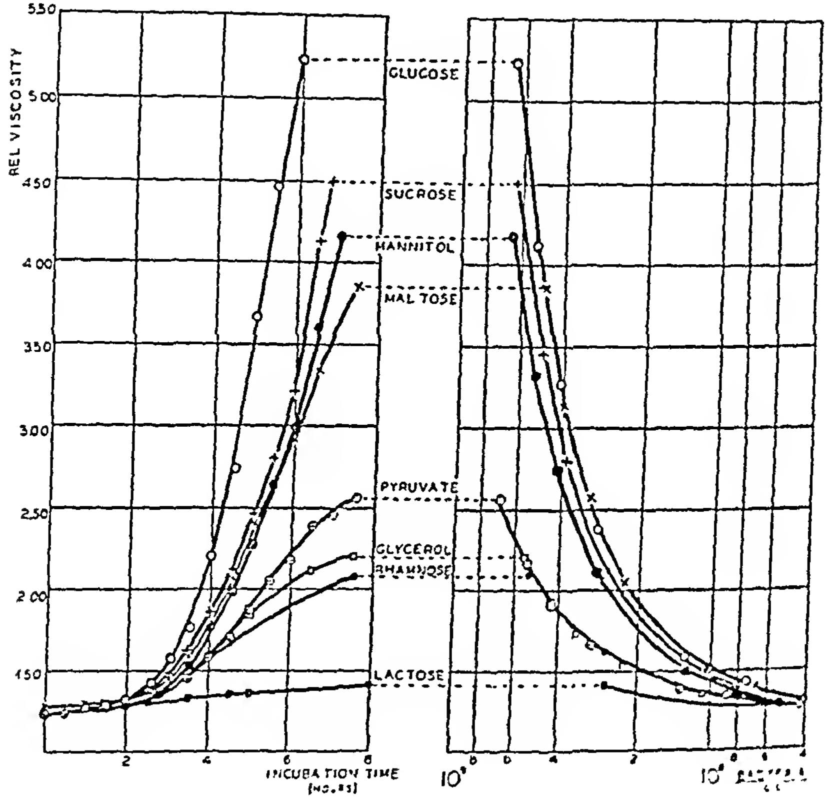

FIG. 5-a   FIG. 5-b

FIG. 5-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone with the addition of 1 per cent of several different fermentable substrates during 8 hours incubation at 37°C. (initial pH 7.5).

5-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

tose, which is practically non-fermentable by the strain used, produces no increase in viscosity.

In order to compare the average size of the capsules in the presence of the different fermentable substrates, dilution curves were made at the end of the experiment. Relative viscosities

for each of the cultures, in relation to the amounts of encapsulated bacteria present, are represented in figure 6-a. It appears from these curves that capsules in the glucose, sucrose, or maltose medium are practically of the same size. In mannitol medium they are somewhat smaller, and in pyruvate, glycerol or rham-

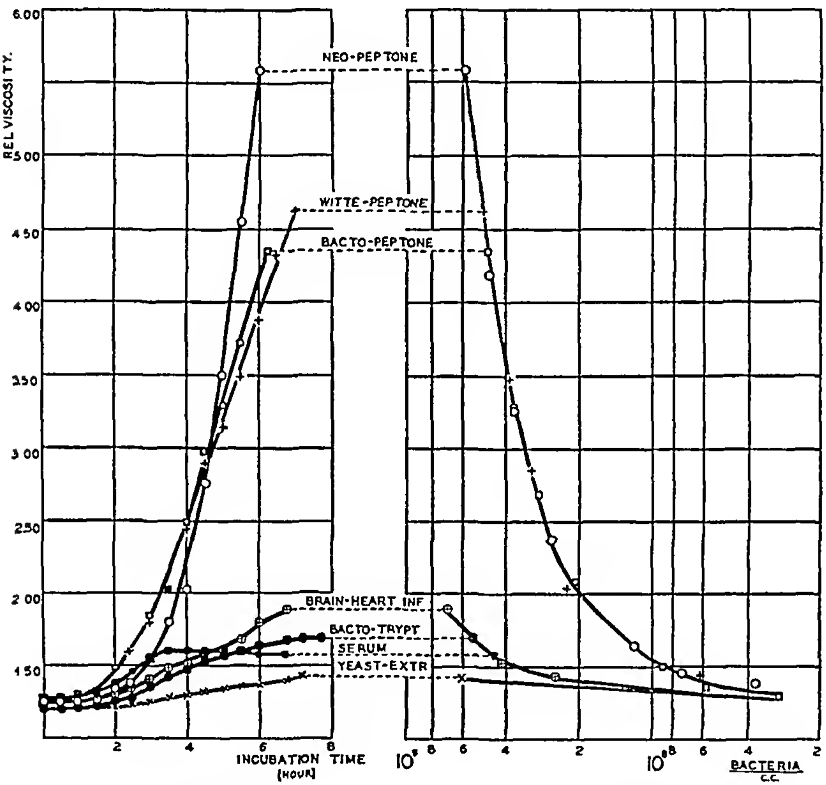

FIG. 6-a FIG. 6-b

FIG. 6-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent solutions of different culture media and 1 per cent glucose during $7\frac{1}{2}$ hours incubation at 37°C. (initial pH 7.5).

6-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

nose medium considerably smaller. It appears also that the capsular substance of Friedländer's bacterium may be synthesized from many different substrates: this is in striking contrast to several non-pathogenic bacteria such as *Betacoccus dextranicus*, which species is able to synthesize its capsules from sucrose only.

INFLUENCE OF THE SOURCE OF PROTEIN ON CAPSULE FORMATION

Higher concentrations of neopeptone favor growth but not capsule formation, inasmuch as capsule size is the same in a ½ per cent as in a 4 per cent neopeptone broth. Other kinds such as (Difco) bactopeptone and Witte peptone give the same result. Growth in these media is somewhat slower, but as may be seen from the dilution curves of figure 6, capsule size in these different media is the same. Unexpectedly, however, many culture media were found in which growth was as good as in neopeptone, but which were completely unsuitable for capsule formation. Four per cent solutions of bactotryptone, yeast extract, Loeffler's blood serum, and brain-heart infusion, all neutralized to pH 7.5, and to which 1 per cent glucose was added, were very favorable culture media for growth, some even better than neopeptone, but capsule formation in these media was extremely poor, as may be seen from the dilution curves in figure 6, and Plate 4. By the addition of increasing amounts of yeast extract to a 2 per cent neopeptone-1 per cent glucose solution, it was found that encapsulation was inhibited; there was greater inhibition with increasing amounts of yeast extract. Apparently yeast extract (and probably also the other unfavorable media) contain some unknown factor which is responsible for this inhibition of encapsulation.

CAPSULE FORMATION IN SYNTHETIC MEDIA

Since Friedländer's bacterium is able to multiply actively in synthetic media of divergent compositions, it was of interest to determine whether capsule formation would occur also in such media. This not only appears to be the case, but capsule size in synthetic media under special conditions may even exceed the size obtained in neopeptone. However, capsule formation in synthetic media is much more delicate than in the ordinary peptone media.

A 2 to 4 per cent solution of asparagin in distilled water containing 1 per cent glucose and neutralized to pH 7.5 does not allow any growth, due to the lack of the required inorganic salts. However, when to this medium are added traces of Na_2HPO_4

(0.0005 to 0.001 per cent) growth becomes possible, but the medium is still far from a favorable one. Bacteria growing under these conditions, however, are surrounded by capsules equal in size or even larger than in the usual neopeptone medium (fig. 7).

When more than traces of Na_2HPO_4 are added (e.g., 0.0012 to 0.0015 per cent or more) growth becomes more pronounced, but encapsulation is very restricted. It also appears that encapsulation in synthetic media takes place only when growth is inhibited by an inadequate amount of phosphate. This fact, of course, is in line with the observation that encapsulation in the 4 per cent

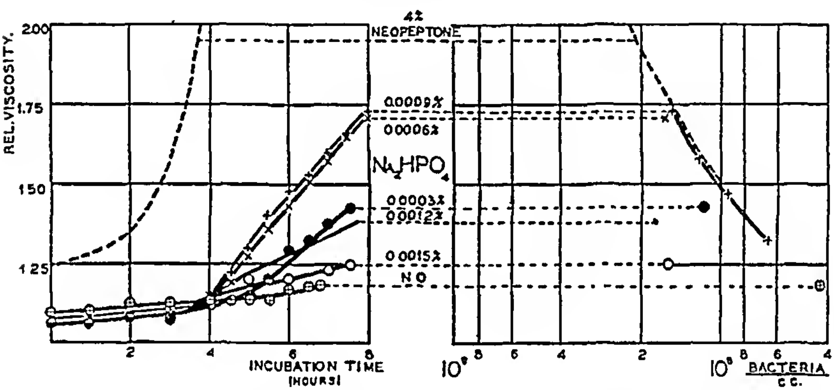

Fig. 7-a Fig. 7-b

Fig. 7-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 3 per cent asparagin-1 per cent glucose solution in distilled water with addition of increasing amounts of Na_2HPO_4 during 8 hours incubation at 37°C. (initial pH 7.5).

7-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

neopeptone-1 per cent glucose medium takes place after the logarithmic growth phase, when growth is restricted due to unfavorable conditions.

Addition of $MgSO_4$ (0.01 per cent), KCl (0.1 per cent) and a $NaHCO_3$ buffer (1 per cent) added either separately or together, favors growth in the 3 per cent asparagin-1 per cent glucose-0.0005 per cent Na_2HPO_4 medium, but there is still a lack of phosphorus and capsule size remains the same. Due to the increased growth, the relative viscosity is much increased by addition of the inorganic salts.

Similar results are obtained when to a 3 per cent asparagin-1 per cent glucose solution traces of neopeptone are added (0.05 to 0.1 per cent). Growth then becomes possible but the medium is still far from favorable. The bacteria, however, are surrounded by capsules of a size larger than in any other medium tested. Phosphate addition (0.01 per cent) or increase in neopeptone

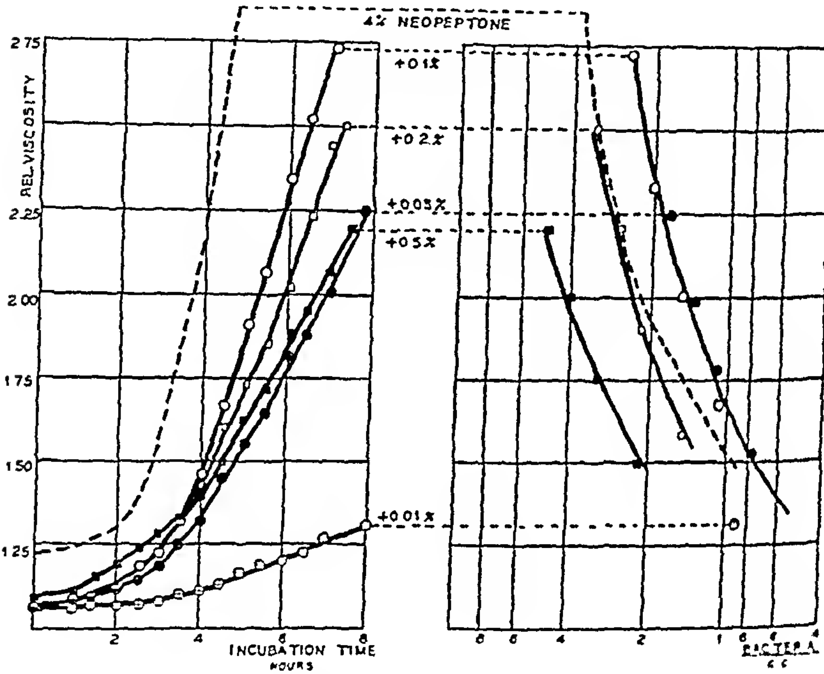

FIG. 8-a FIG. 8-b

FIG. 8-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 3 per cent asparagin-1 per cent glucose solution in distilled water with the addition of increasing small amounts of neopeptone. The same for cultures in 4 per cent neopeptone-1 per cent glucose medium.

8-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

concentration (0.1 to 0.5 per cent) favors growth, but makes the medium less suitable for encapsulation (fig. 8).

These observations once more stress the fact that the conditions under which capsules are produced are not identical with the conditions under which maximal growth occurs; on the contrary, there seems to exist a contra-relation between growth and en-

capsulation, inasmuch as capsule production seems to occur mainly under conditions unfavorable for growth.

INFLUENCE OF THE pH OF THE MEDIUM AND OF THE INCUBATION TEMPERATURE ON ENCAPSULATION

Friedländer's bacterium is able to grow over a very wide pH range, from about pH 5.0 to pH 9.0, its optimal growth being at

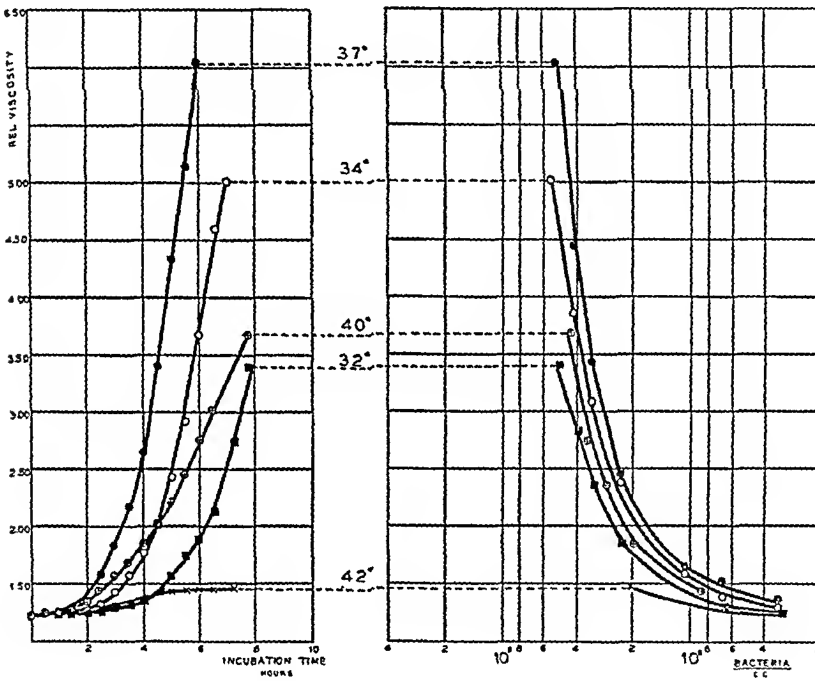

FIG. 9-a FIG. 9-b

FIG. 9-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose incubated at varying temperatures (32–42°C.) (initial pH 7.5).

9-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

pH 7.5. It appears, however, that encapsulation is practically independent of the pH of the medium. The same capsule size was obtained in 4 per cent neopeptone-1 per cent glucose, neutralized to pH 6.5, 7.0, 7.5 and 8.0. When not otherwise stated, in all experiments the medium was neutralized to pH 7.5.

Friedländer's bacterium will develop over a wide range of

temperature up to 42°C. Its optimum temperature is 37°C. A sharp drop is observed above 37°C. Below 37°C., growth is retarded but the decline is a gradual one. The same may be said for the increase in relative viscosity. Growing in 4 per cent neopeptone-1 per cent glucose at pH 7.5, the greatest increase in viscosity is observed when incubated at 37°C. At 40°C., this increase is markedly retarded, and at 42°C. almost no increase in viscosity is observed (fig. 9). Capsules of bacteria grown at 40°C., and especially at 42°C., are much smaller than at 37°C. It is probable that at higher incubation temperatures, sugar is fermented to a greater extent (more acid is formed) and less synthesis to polysaccharide takes place. At temperatures lower than 37°C., capsule size diminishes gradually with decrease in temperature, at 32°C. the size being considerably smaller than at 37°C. (*When not otherwise stated all experiments have been* performed at 37°C.)

THE INFLUENCE OF BUFFER IN THE MEDIUM

A. $NaHCO_3$

The addition of $NaHCO_3$ up to 1 per cent to the nutrient broth increases growth markedly, probably by neutralizing part of the acids formed during fermentation. Viscosity in the presence of $NaHCO_3$ increases tremendously, 5 hours after inoculation the relative viscosity having increased to a value of almost 12 (fig. 10).

Since capsule size is not at all, or only slightly, affected by the addition of $NaHCO_3$ such an addition may be advocated, for instance, in the isolation of capsular polysaccharides from cultures of Friedländer's bacterium.

B. Na_2HPO_4

The addition of Na_2HPO_4 to the nutrient broth also causes a marked increase in growth and viscosity compared with the phosphate-free culture. However, this increase is less than that caused by $NaHCO_3$. A slight precipitate is formed, probably of Ca-phosphate. When this precipitate is not removed, capsule size is the same as in the medium without phosphate, although

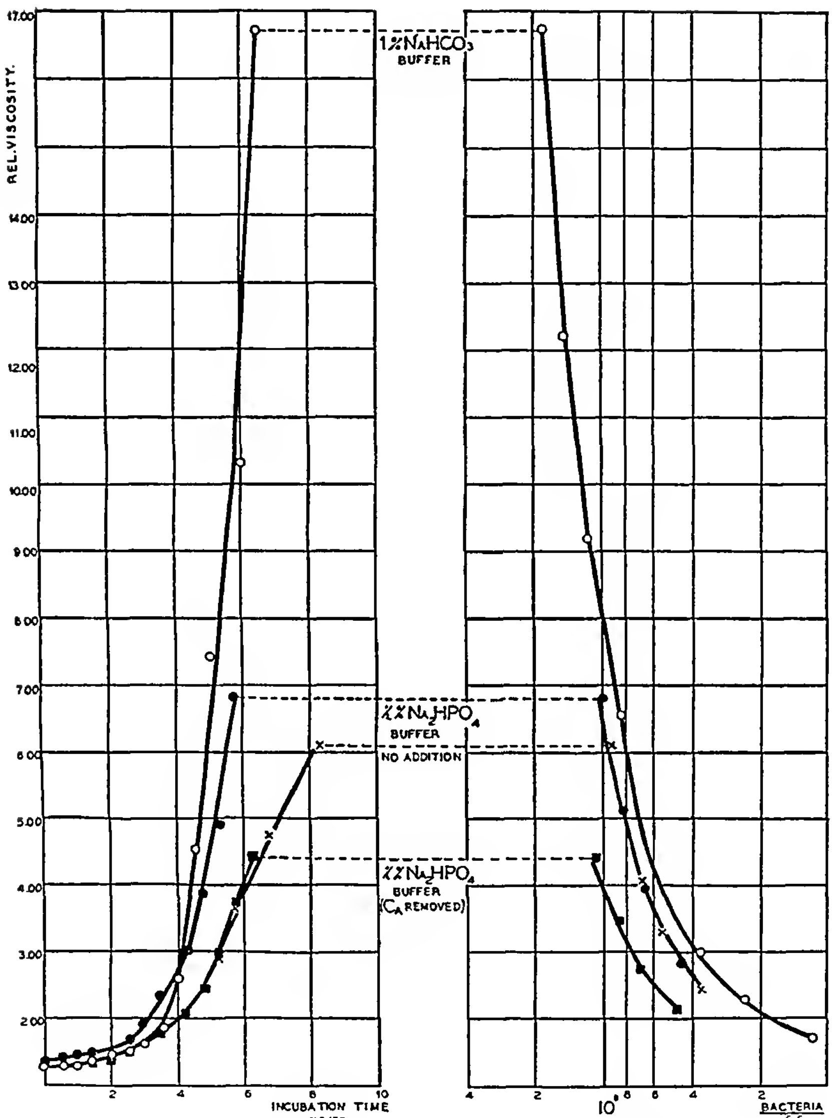

FIG. 10-a

FIG. 10-b

FIG. 10-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose medium with and without addition of carbonate or phosphate buffer during 8 hours incubation at 37°C. (initial pH 7.5).

10-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

the viscosity is much higher, due to the better growth. When, however, this precipitate is removed from the culture medium, a marked inhibition of capsule formation is observed. This inhibition is due to the removal of practically the entire calcium content of the culture medium. This was proved to be true by a preliminary treatment of the culture medium with Na oxalate in such amount that all of the calcium originally present was precipitated. Capsule formation in such a medium deprived of calcium is much inferior to that in the original medium, but by adding calcium in the form of calcium chloride in the amount that originally was present, capsule formation becomes normal. This indicates that calcium may play an important role in the formation of capsular substance.

IS HEAVIER ENCAPSULATION DUE TO SWELLING OF THE CAPSULES OR TO AN INCREASED PRODUCTION OF CAPSULAR SUBSTANCE?

The question arises whether varying capsule size is really due to an increased production of capsular material or whether it can be ascribed to a greater or lesser swelling of the capsules. This possibility must be taken into consideration, since it is possible to demonstrate a marked swelling of capsules in general by addition of suitable amounts of antiserum (Neufeld reaction). This reaction, however, is specific inasmuch as it can be brought about only by type-specific antisera. Its mechanism is entirely unknown. Consequently the amount of capsular material produced in several different culture media was determined by the combining equivalent method of Felton and Stahl (1935).

Cultures of Friedländer's bacterium were prepared in 4 per cent neopeptone with and without addition of 1 per cent glucose, and also in 4 per cent yeast extract-1 per cent glucose. These cultures were incubated for 7 hours, after which counts were made to determine the bacterial population. Each culture was then diluted with saline until the bacterial concentration was 10^{9} bacteria per ml. The cultures were subjected to a 10-minute treatment with ultrasonic vibration, which brings the capsules completely into solution.

Dilution series of these polysaccharide solutions were prepared and 0.3 ml. of each dilution were mixed with 0.3 ml. of antiserum. It was then determined which dilution contained just enough polysaccharide to combine with all the antibodies present in a standard amount of anti-serum. A culture of 4 per cent neopeptone without glucose (10^8 bacteria per milliliter) had to be diluted 1:2; the culture in 4 per cent yeast extract—1 per cent glucose had to be diluted 1:4; a three-hour culture in 4 per cent neopeptone—1 per cent glucose, (10^8 bacteria per milliliter) also had to be diluted 1:4; however, a seven-hour culture in 4 per cent neopeptone—1 per cent glucose had to be diluted 1:16 in order to precipitate all of the polysaccharide present.

This makes it clear that the amount of polysaccharide produced per bacterium in 4 per cent neopeptone—1 per cent glucose, was about 4 times that in 4 per cent yeast extract, and about 8 times that in 4 per cent neopeptone without glucose.

Since the results of these polysaccharide determinations on cultures in various media are in agreement with the viscosity measurements, and with the sizes of capsules as demonstrated by India ink smears, it seems, that heavier encapsulation means greater production of polysaccharide and not merely swelling of the capsules.

Similar results were obtained by estimation of the dry weight of the precipitated bacteria from 20 ml. of cultures of Friedländer's bacterium in the media mentioned above, after precipitation with 20 ml. of alcohol. The highest dry weight for a standard amount of bacteria was obtained from a seven-hour culture in 4 per cent neopeptone—1 per cent glucose.

DISCUSSION

Many investigators are of the opinion that heavy capsule formation takes place only in the animal body as a protection against phagocytosis, that continued subculturing on artificial media gives rise to a gradual diminution in capsule formation, and that repeated animal passages may completely or partially restore the loss.

As a matter of fact, there is little reason to assume that body fluids would possess such a magical action by which pathogens could encapsulate themselves. This study indicates that the reason why many encapsulated organisms lose their capsules by continued subculturing on artificial media may be that the media upon which they are cultivated contain such abundance of nutrient substances that there is no tendency towards encapsulation.

In many cases, it has been shown that encapsulation occurs under conditions unfavorable for growth and not when active proliferation is occurring. Instead of transplanting freshly isolated mucoid strains frequently, it seems more reasonable to store them as long as possible and to transplant them no oftener than is absolutely necessary.

The abundance with which Friedländer's bacterium forms capsular polysaccharides under certain circumstances offers no reason to believe that the capsule is a cell constituent; it is more reasonable to consider it as a metabolite. Under certain conditions the normal bacterial metabolism is changed and instead of the normal assimilation to new cell-material (growth), abnormal assimilation takes place in which polysaccharides are synthesized from the intermediates of the sugar break-down.

To what extent the formation of capsules only after the logarithmic phase of growth is a general phenomenon is not known, but it may be stated that *Betacoccus dextranicus* (*Leuconostoc mesentericus*) behaves similarly to Friedländer's bacterium in this respect.

SUMMARY

1. Friedländer's bacterium was grown in Ostwald viscosity tubes, and, from the determinations of relative viscosity and simultaneous bacterial counts, deductions could be made as to the degree of encapsulation.

2. The largest capsules were obtained by cultivation for about 8 hours at 37°C. in a medium containing 1 to 4 per cent peptone and 1 per cent glucose, initial pH 7.5.

3. Capsule formation took place mainly after the logarithmic

phase of growth when the multiplication of the bacteria became restricted.

4. In the absence of sugars, the capsules were very small. The presence either of glucose, maltose, sucrose, mannitol, or to a lesser extent, pyruvate or glycerin, was necessary to ensure the formation of large capsules.

5. The protein source was also important for encapsulation. Neopeptone, Witte peptone or bactopeptone served equally well as sources of protein. However, several media such as yeast extract, Loeffler's blood serum, and brain-heart infusion contained factors, as yet unknown, which inhibited encapsulation markedly, although they did not affect the growth of the bacteria.

6. In a synthetic medium, capsule formation took place only under special conditions, i.e., when the phosphate concentration was below the limits of optimal bacterial growth.

The author wishes to express his gratitude to Dr. Ellice McDonald, Director, for his interest and support throughout this work.

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PLATES 1-4

India Ink Smears of Encapsulated Friedländer's Bacterium Grown under Different Conditions

Plate 1. Encapsulated bacteria from a 4 per cent neopeptone culture without addition of carbohydrates.

Plate 2. Encapsulated bacteria from a 4 per cent neopeptone-1 per cent glucose culture (3 hours after incubation).

Plate 3. The same culture 7 hours after incubation.

Plate 4. Encapsulated bacteria from a 4 per cent yeast extract-1 per cent glucose culture.

PLATE 1

PLATE 2

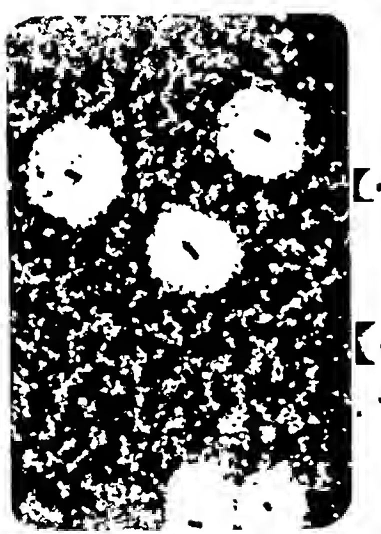

PLATE 3

PLATE 4

(J. C. Hoogerheide)

THE EVALUATION OF GERMICIDES BY THE MANOMETRIC METHOD

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The use of manometric methods in the evaluation of germicides has received comparatively little consideration in the past. Bronfenbrenner, Hershey and Doubly (1938) have reported the use of such a method based on a 50 per cent inhibition of oxygen consumption by a bacterial suspension during an arbitrarily chosen interval between the 15th and the 20th minute.

This paper presents data represented by the accompanying graphs bearing on the following questions relating to the use of the manometric method in the evaluation of germicides:

1. The relation between the inhibition by germicides of oxygen consumption of a bacterial suspension and the subsequent ability of the suspension to grow when subcultured.
2. The effect of germicides on oxygen consumption in relation to time.
3. A comparison of the amount of inhibition of oxygen consumption caused by several germicides on bacterial suspensions and the relative number of organisms killed.
4. A comparison of the effect of several germicides on bacteria suspended in a synthetic medium and in rabbit serum.

METHODS

Escherichia coli was used as the test organism. Eighteen- to twenty-hour cultures on agar slants of Bacto heart-infusion broth were suspended either in rabbit serum or in the synthetic medium described below, a uniform suspension being obtained by filtration through a pad of cotton to remove clumps. Oxygen consumption was measured by the usual Warburg technic at 37.5°C.

Following the manometric determination, subcultures were made in heart-infusion broth.

A typical experiment is as follows: 1.1 ml. of the bacterial suspension was placed on the floor of each of the reaction vessels and 0.4 ml. of 4N-NaOH in the well (with a strip of filter paper) for carbon dioxide absorption. The germicidal solution was placed in the side arms of the vessels except for the control, in which distilled water was substituted. After an initial period of observation during which the respiration of the samples was uniform, the germicide was added from the side arm and observations made at suitable intervals. This technic was varied slightly in some experiments. It was found advantageous, in some cases, to add the germicide directly to the bacterial suspension at the beginning of the determinations.

The synthetic medium used, and hereafter designated as S. Medium, had the following composition.

Dipotassium phosphate	0.05 grams
Magnesium acid phosphate	0.05 grams
Ferric citrate	0.005 grams
Glucose	2.00 grams
Sodium citrate	0.25 grams
Ammonium sulphate	0.30 grams
Calcium nitrate	0.025 grams
Distilled water	100.00 cc.
Sodium hydroxide to	pH 7.4

plate cultures showed the presence of less than 1% of foreign organisms.

EXPERIMENTAL RESULTS

1. The relation between the inhibition by germicides of oxygen consumption and the subsequent cultural results

The number of organisms per ml. in the suspensions used in this series of experiments was not constant, the growth from one slant suspended in 7 to 10 ml. of S. medium giving approximately 3.5×10^9 to 4.5×10^9 organisms per ml.

The results indicate that so long as a suspension of bacteria shows any trace of oxygen consumption it contains viable bacteria but when the oxygen uptake is reduced to zero the suspension contains only dead organisms. Figure 1 shows a typical example. Curve 4 in figure 1 shows that although the respiration may be inhibited almost completely positive subcultures still result. Similar results were obtained in a number of other experiments not shown here. In the figures a positive culture is represented by a plus (+) sign, a negative culture by a minus (−) sign. From these results it appears safe to assume that all of the organisms are killed when the oxygen uptake becomes zero. Figure 2 confirms this assumption showing that at the end of a 15-minute period, cultures were positive if the oxygen consumption was still evident during the period, but negative if the oxygen consumption had been zero during the period.

2. The effect of germicides on oxygen consumption in relation to time

The results of any of the experiments may be used for the preparation of data relating to this question by calculating the percentage of inhibition of oxygen consumption during successive observation periods. The data for successive half-hour periods for several experiments are represented by figure 3. This figure shows that the percentage inhibition increases with time in each case until a maximum is attained. If the degree of inhibition reaches 100 per cent the culture is dead; if below 100 per cent the inhibition remains at the maximum for successive periods.

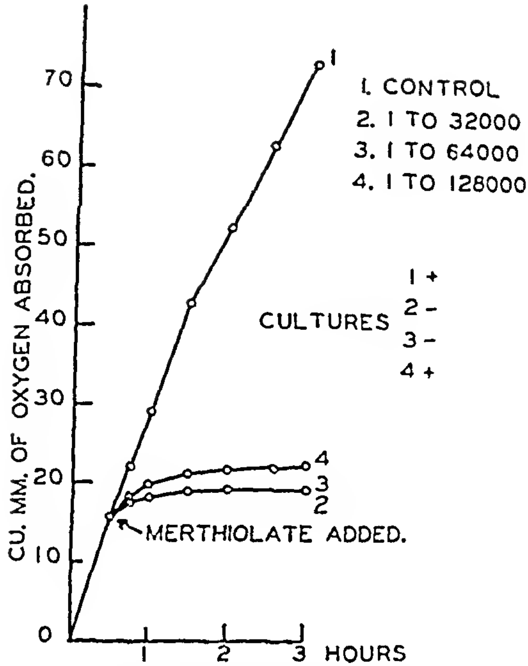

FIG. 1. THE EFFECT OF AQUEOUS MERTHIOLATE ON E. COLI IN S. MEDIUM

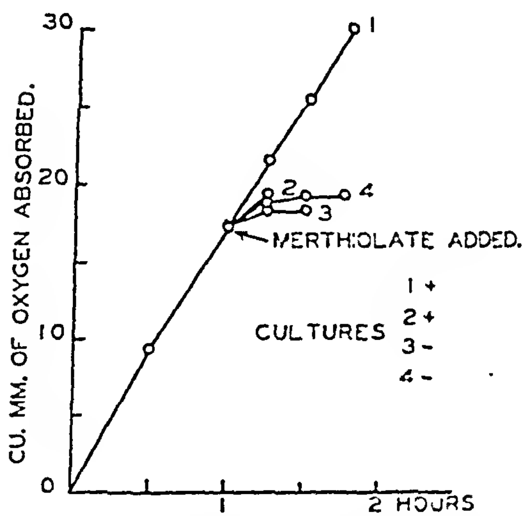

FIG. 2. EFFECT OF AQ. MERTHIOLATE 1:24,000 ON E. COLI IN S. M.
Oxygen uptake and cultural results at 15 minute intervals

In no case did the amount of inhibition recede from the maximum, except in amounts which might be ascribed to experimental error.

3. A comparison of the amount of inhibition of oxygen consumption caused by several germicides on a bacterial suspension and the relative number of organisms killed

(a) *E. coli*, 2.6 × 10^9 organisms were suspended in a dilution of 1:100 of sulfanilamide in S. medium. During a period of 1.25 hours the respiration had been inhibited 55 per cent with reference to the control, with very little, if any, reduction in the number of living organisms as shown by plate cultures.

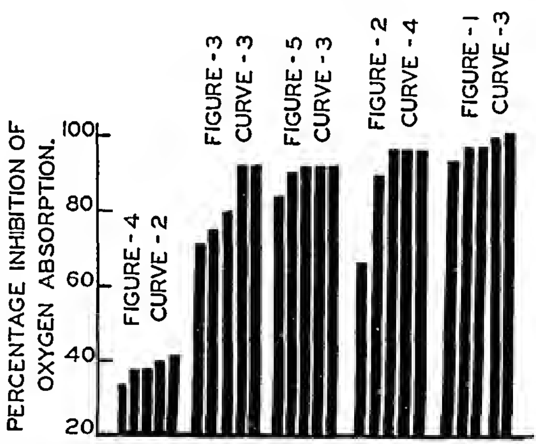

FIG. 3. THE PERCENTAGE INHIBITION OF OXYGEN ABSORPTION DURING SUCCESSIVE HALF-HOUR PERIODS

(b) *E. coli*, 1.7 × 10^9 organisms were suspended in 1:120 sulfanilamide in rabbit serum (serum 5 parts + distilled water 13). During a period of 1.25 hours the respiration of the bacterial suspension had been inhibited 50 per cent with no reduction in the number of living organisms in comparison with the control suspension.

(c) *E. coli*, 2.6 × 10^9 organisms were suspended in 1:128,000 merthiolate in S. medium. In 15 minutes the respiration had been inhibited 61 per cent and the number of living organisms reduced by 43 per cent with reference to the control.

(d) *E. coli*, 1.7×10^9 organisms were suspended in merthiolate 1:9500 in rabbit serum. The respiration was inhibited 33 per cent in a 30 minute period. It was then found that the number of living organisms had been reduced by only 7 per cent.

4. A comparison of the effect of several germicides on bacteria suspended in S. medium and in rabbit serum

In this series of experiments the final serum suspending medium (after the addition of the germicide solution) was composed of 0.55 ml. of serum and 1.05 ml. of distilled water in each reaction vessel. In each experiment the number of bacteria suspended in the S. medium and in the serum was the same.

The germicidal effects of merthiolate, sulphated castor oil[1] containing 20 per cent sodium o-phenyl-phenate, and tincture of iodine were greatly reduced in the presence of serum. Typical of these results are those shown by figure 4 for merthiolate. Tincture of iodine killed the culture (1.9×10^9 bacteria per ml.) in approximately 15 minutes in S. medium but caused in serum only a 74 per cent inhibition in respiration at the end of 30 minutes. Sulphated castor oil containing sodium o-phenyl-phenate caused only a 32 per cent inhibition of respiration in a suspension in serum containing 2.2×10^9 bacteria per ml. during a period of 1.25 hours following its addition to the suspension, whereas in S. medium the culture was killed in approximately 15 minutes. Merthiolate caused, in serum, only a 10.5 per cent inhibition of respiration in a period of 2.75 hours following its addition to the suspension as compared with a 78 per cent inhibition when in S. medium (fig. 4).

The germicidal power of sulfanilamide was unaffected by serum, showing in 4 hours 45 per cent inhibition of respiration in serum and 40 per cent inhibition in S. medium (fig. 5).

Formaldehyde (U.S.P.) in a concentration of 1:800 caused a 66 per cent inhibition of respiration in serum during a period of

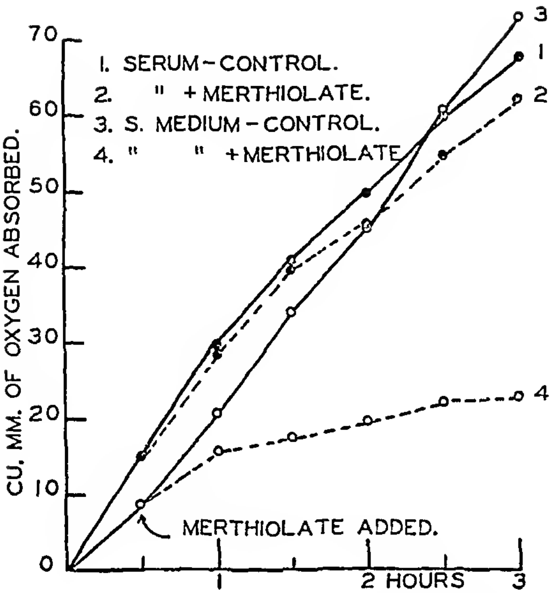

FIG. 4. A COMPARISON OF THE EFFECT OF AQUEOUS MERTHIOLATE ON E. COLI IN S. MEDIUM AND IN RABBIT SERUM

Concentration of methiolate 1:128,000. Bacteria per cubic centimeter 3.1 $\times 10^9$.

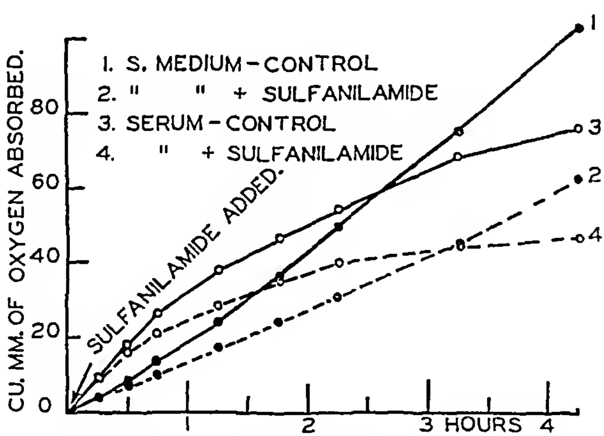

FIG. 5. COMPARISON OF THE EFFECT OF SULFANILAMIDE ON E. COLI IN S. MEDIUM AND IN RABBIT SERUM

Concentration of sulfanilamide 1:122. Number of bacteria per cubic centimeter 2.8 $\times 10^9$.

30 minutes and a 76 per cent inhibition in S. medium (1.7×10[illegible] bacteria per ml.). Phenol in a concentration of 1 to 250 caused a 72 per cent inhibition in respiration over a period of 2.25 hours in serum and a 75 per cent inhibition in S. medium (2.7×10[illegible] bacteria per ml.).

DISCUSSION

The germicides, in the concentrations used and under the conditions of these experiments, caused varying degrees of inhibition of the respiration of *E. coli* with a lethal effect if the inhibition of respiration was 100 per cent. The evidence presented under section 3 indicates that with sulfanilamide, a respiratory inhibition of *E. coli* in S. medium, or in rabbit serum, of 55 per cent or less, may be attained with little or no killing of the bacteria. Whether or not a higher concentration of this germicide, causing a greater inhibition of respiration, would prove lethal to some of the bacteria of the suspension has not been determined.

Aqueous merthiolate, in contrast to sulfanilamide, was lethal to some of the organisms when only a 33 per cent inhibition of respiration was reached and to a much greater extent when a 61 per cent inhibition of respiration had occurred. It would appear from these results that the effect on *E. coli* of an aqueous solution of merthiolate may differ somewhat from that [illegible] fanilamide.

It seems reasonable to assume that merthiolate, and pr[illegible] some of the other germicides as well, affected the respirat[illegible] all of the organisms in the suspensions and killed first the [illegible] members of the population. This assumption is based [illegible] fact that the percentage inhibition of respiration cau[illegible] merthiolate was much greater than the relative percentage [illegible] organisms in the suspension which were killed, and presu[illegible] that the respiration of one bacterium is equal to that of an[illegible] which it must be admitted may not be true.

Apparently, it is necessary that a germicide suppress th[illegible] piration of a bacterial suspension to a considerable extent [illegible] any lethal effect occurs. For example, merthiolate was fou[illegible]

have killed only 7 per cent of the living organisms of a suspension in a 15-minute period during which the respiration had been inhibited by 33 per cent. It seems probable that a sufficiently low concentration of germicide might show some inhibition of respiration with no killing effect whatever.

Merthiolate in a concentration which produced an inhibition of respiration of 61 per cent killed 43 per cent of the organisms of a suspension, whereas sulfanilamide in concentrations which produced in one case a 55 per cent inhibition of respiration and in another case a 50 per cent inhibition had little or no killing effect. The question naturally arises as to the accuracy of phenol coefficients of germicides determined by a comparison of their effects on the oxygen consumption of bacterial suspensions with that of phenol. If comparable amounts of inhibition of respiration by different germicides indicate that comparable numbers of the bacteria have been killed, the phenol coefficients so determined should be accurate. However, it is not certain at present that this is the case. Further investigation of this question seems necessary since differences in this respect between germicides other than merthiolate and sulfanilamide may be found.

SUMMARY AND CONCLUSIONS

1. Manometric studies of the effects of several germicides on the oxygen consumption of *Escherichia coli* in a synthetic medium have been made and compared with the lethal effects as shown by subsequent cultures. A complete inhibition of respiration was found necessary to render the organisms incapable of growth when subcultured. If even a slight respiration was evident positive cultures resulted. On the other hand negative cultures always resulted as soon as the respiration reached zero.

2. When germicides were added to suspensions of *E. coli* the percentage of inhibition of respiration increased during each successive observation period until a maximum was reached. In no case did the effect recede from the maximum.

3. The relation between the degree of inhibition of respiration and killing by sulfanilamide and by merthiolate was found to differ in that the amount of inhibition of respiration by sulfanil-

amide without a killing effect was greater than that for merthiolate.

4. The presence of serum greatly inhibited the germicidal effect of merthiolate, tincture of iodine and sulphated castor oil containing sodium o-phenyl phenate, had little or no effect on sulfanilamide, and only a moderate effect on phenol and formaldehyde.

The author wishes to express his appreciation to Dr. Ellice McDonald, Director, for his interest and support throughout this work.

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INVESTIGATIONS UPON THE ANTIGENIC RELATIONSHIPS AMONG THE ROOT-NODULE BACTERIA OF THE SOYBEAN, COWPEA, AND LUPINE CROSS-INOCULATION GROUPS[1]

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Although several workers have investigated the serological characteristics of the organisms in the genus *Rhizobium*, few have given much consideration to the possible relationships between bacteria isolated from plants of the soybean, cowpea, and lupine groups. Moreover, none of these workers has had the benefit of recent discoveries in immunology, which have made new concepts and new techniques available for studies of the antigenic constitution of bacteria.

The frequency with which recent papers (Leonard, 1923; Sears and Carroll, 1927; Hansen and Tanner, 1931; Carroll, 1934; Walker and Brown, 1935; Raju, 1936; Reid, 1936; Conklin, 1936; Toxopeus, 1936; Bushnell and Sarles, 1937) have reported "inter-crossings" among the bacteria and plants of the soybean, cowpea, and lupine groups, and the consequent confusion attending upon the classification of both plants and bacteria in these groups, has made advisable some study of the antigenic constituents of these bacteria. Such a study must necessarily consider the roles played by flagellar and by capsular substances in determining the serological specificity of a bacterial strain. The agglutination test appeared to lend itself best to a study of this sort.

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METHODS

Antisera were prepared with strains of bacteria obtained from plants in each of the three groups. The strains were so selected that some formed nodules as readily on soybean as on cowpea and lupine plants, while others formed nodules on two, but not on all three, of the host plants. The ability of these strains to infect the different host plants was established by Reid (1936) in a number of consecutive greenhouse experiments made under the wide range of variation in length of day experienced in Madison, Wisconsin. The cross-inoculation relationships of the strains used as antigens are given in table 2.

Preparation of antigens

The organisms were grown on slants of yeast-water mannitol mineral-salts agar medium containing potato extract, which supplies an accessory growth factor (Sarles and Reid, 1935) favoring both the growth and production of capsular polysaccharides of the organisms from soybeans, cowpeas, and lupines. After 5 days at 28°C., the growth was washed from the agar and suspended in 0.5 per cent sodium chloride solution.

From each strain three antigens were prepared:

1. A suspension of the untreated cells was used as the "whole-cell antigen," referred to as the "whole-antigen."
2. A suspension of cells, heated for two hours in flowing steam to inactivate the "flagellar" proteins, provided the "heated antigen."
3. Cells believed to be capsule-less were obtained by treating the suspensions with warm 0.5 per cent saline solution at 65°C., (Hopkins, Peterson, and Fred, 1930) followed by centrifugation. Three successive washings with warm saline solution removed the gum, and the "flagellar" proteins were inactivated by heating the capsule-free cells in flowing steam for two hours. This capsule-less, flagella-less form of antigen is called, for want of a better term, the "washed-heated antigen," or "washed-antigen."

Preparation of immune sera

Each of the antigens, suspended in 0.5 per cent sodium chloride solution, was then injected intraperitoneally at intervals of 5

days into a separate rabbit. The dose increased from 1.0 ml. of the stock suspension at the first injection to 2.0 ml., then 3.0 ml., and finally to 3.5 ml. at the fourth and last injection. One week after the last injection each rabbit was bled from the ear; the blood was placed in the refrigerator for 24 hours; then the serum was removed and stored at 4°C. No preservative was used, except in the sera for Soybean strain 10, Cowpea strain 602, and Lupine strain 16,400 which were preserved with 0.5 per cent phenol.

Sera were prepared against antigenic modifications of 10 different strains of root-nodule bacteria: 4 from soybeans, 3 from cowpeas, and 3 from lupines. In some instances, in order to introduce more strains into the study, only 2 antigenic modifications of the strains were used; (in one other the rabbit being immunized died a few days before it was to have been bled, so that the serum it was to have provided was lost). In all, 25 different antisera were obtained, and are listed below:

WHOLE-CELL	HEATED	WASHED-HEATED
Soy 10	Soy 10	Soy 10
Soy 505	Soy 505	Soy 505
Soy 500	Soy 500	
Soy 507	Soy 507	
Cowpea 601	Cowpea 601	Cowpea 601
Cowpea 602	Cowpea 602	Cowpea 602
	Cowpea 603	
Lupine 804	Lupine 804	Lupine 804
Lupine 810	Lupine 810	
Lupine 16,400	Lupine 16,400	Lupine 16,400

Agglutination tests

These antisera were employed in simple cross-agglutination tests against the three modifications of "whole-cell," "heated," and "washed-heated" antigens prepared in the same manner as were the immunizing antigens from 54 strains of root-nodule bacteria from plants in the soybean, cowpea, and lupine cross-inoculation groups.

Whole-cell antigens prepared from rhizobia from alfalfa, clover, and pea plants were also tested against these antisera.

The test-antigens were suspended in 0.5 per cent saline solution containing 0.5 ml. of a 40 per cent solution of formaldehyde per 100 ml. of antigen. The dilutions of serum were also prepared with a similar salt-formalin solution in order to prevent growth of organisms in the lower dilutions during the period of incubation of the tests. The sera were used in dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:2000, 1:3200, 1:4000, and 1:6400; sometimes other dilutions were interpolated in the lower ranges or added at the upper limits. The tubes were allowed to incubate 18 to 20 hours at 37°C. before the titers were recorded. Each test was performed at least twice.

RESULTS AND COMMENT

The data presented in the tables are those which provide the most information with the least amount of detail; the rest of the data obtained, while bearing out the general conclusions drawn from the cited figures, are not included because to present them would provide too cumbersome and too confusing a compilation.

The most striking feature of the results given in table 1 is the large number of "serological types" found among the strains studied. These results confirm those of Stevens (1923) who concluded that the agglutination test can not be used to identify all strains of a single species of the root-nodule bacteria. The antigenic constitution of different strains within a species is apparently dissimilar; hence an antiserum for one strain of, for example, *Rhizobium japonicum*, will not agglutinate all strains of that species. The many minus signs in this table, each marking an instance where there was no clumping of the test antigen by the serum employed, offer testimony to this limitation of the agglutination reaction.

The success of Lancefield (1933) and of Plummer (1935) in their serological classification of the streptococci by means of the precipitation reaction suggests that perhaps that method is better suited than is the agglutination reaction for distinguishing the species antigen as well as the more variable type-antigens

TABLE 1

Serological types: highest dilution at which clumping of whole-cell test-antigens was obtained with antisera prepared against whole-cells

WHOLE-CELL TEST ANTIGENS	ANTISERA PREPARED AGAINST WHOLE-CELL ANTIGENS OF:								
	Soy 505	Soy 10	Soy 500	Soy 507	Cowpea 601	Cowpea 602	Lupine 804	Lupine 810	Lupine 16,400
Rhizobium japonicum:									
Soy 8			1,600				400	400	
Soy 10	800	6,400	2,000	200	400	3,200	400	200	200
Soy 500	800		6,400						
Soy 505	6,400							800	
Soy 507				4,000			2,000		
Soy 508	400			400	100		4,000		
Soy 510	400	200	3,200				1,600		
Soy 516							400		
Rhizobium for cowpea:									
Cowpea 600					4,000				
Cowpea 601	2,000		100		4,000		100	2,000	2,000
Cowpea 602	800				100	100	400		
Cowpea 603	50		800						
Cowpea 605	6,400		100	100		200	100	400	200
Cowpea 606	6,400			200	4,000	400	4,000	200	
Cowpea 610							100		
Cowpea 617	800	800	800	400	400	4,000	3,200		
Rhizobium lupini:									
Lupine 801				100			800		
Lupine 804						100	1,600		
Lupine 807		200	400		100		100		100
Lupine 810	800		800				100	4,000	200
Lupine 812			100						
Lupine 16,400							200		12,800
Rhizobium meliloti									
Rhizobium trifolii									
Rhizobium leguminosarum									

In tables 1 and 2, blank spaces indicate negative results.

in the bacterial complex. This approach to the problem must yet be tried.

Just as obvious as are the serological types within a species,

TABLE 2

Relation between cross-inoculation and cross-agglutination

STRAIN	CROSS-INOCULATION; NODULES FORMED ON:			CROSS-AGGLUTINATION: HIGHEST DILUTION AT WHICH CLUMPING OF WHOLE-CELL ANTIGENS WAS OBTAINED WITH ANTISERA PREPARED WITH WHOLE CELLS:									
				Sera for Soybean Strains				Sera for Cowpea Strains			Sera for Lupine Strains		
	Soybeans	Cowpeas	Lupines	500	505	507	10	601	602	603*	804	810	16,400
Rhizobium japonicum:													
Soy 500	+	+	−	6,400	800					2,000			
Soy 505	+	+	−		6,400							800	
Soy 507	+	+	−			4,000					2,000		
Soy 10	+	+	−	2,000	800	200	6,400	400	3,200	100	400	4,000	200
Rhizobium for cowpea:													
Cowpea 601	+	+	−	100	2,000			400		3,200	100		2,000
Cowpea 602	−	+	+		800			100	12,800	100	400		
Cowpea 603	+	+	+	800	200					6,400			
Rhizobium lupini:													
Lupine 804	+	+	+		100				100		2,000		
Lupine 810	−	+	+	800	800						100	4,000	200
Lupine 16,400	−	−	+								200		12,800
Rhizobium meliloti	−	−	−										
Rhizobium trifolii	−	−	−										
Rhizobium leguminosarum:	−	−	−										

* Antiserum prepared with heated cells.

however, are the many instances in which antisera prepared against a strain of rhizobia from one cross-inoculation group caused agglutination of bacteria from the other two cross-inoculation groups as well. The serum of Lupine strain 804 is distinctive for its ability to agglutinate almost all strains of soybean bacteria that were tested, and the majority of the strains from plants in the cowpea group. The fact that the sera for Lupine strain 810, Cowpea strains 601 and 602 and Soybean strains 505 and 507 possess to some degree this same ability to agglutinate strains of other species makes it likely that this faculty is not a characteristic peculiar to Lupine strain 804, but that it is shared to a greater or lesser degree by many of the strains of root-nodule bacteria from these three cross-inoculation groups. It is noteworthy, however, that there is less cross-agglutination, just as there is less cross-inoculation (as shown in table 2) between strains from the soybean and lupine groups than there is between strains from either of these two groups with those from the cowpea group.

There seems to be no correlation between the ability of the strains to cross-inoculate and to cross-agglutinate. For instance, soybean strain 505, which forms nodules on both soybean and cowpea plants, but not on lupine plants, is not agglutinated by any of the antisera for cowpea bacteria, but is agglutinated to a fair titer by the serum for one strain of *Rhizobium lupini*. Soybean strain 500, which forms nodules on both soybean and cowpea plants, is agglutinated by sera from some strains of the soybean *Rhizobium*, but the serum from only one of the strains of the *Rhizobium* for cowpea will cause its agglutination. Another instance of this sort is that of Cowpea strain 603, which, although it forms nodules on plants in all three cross-inoculation groups, reacted with no other sera of the cowpea group, and only fairly well with the sera from two strains of soybean rhizobia.

On the other hand, strains which form nodules on plants in at least two of the three cross-inoculation groups, were agglutinated by sera for strains of bacteria derived from all three of the groups. Soybean strain 10, and Cowpea strains 601 and 602 are examples of this sort. Apparently the serological nature of the strains of

soybean, cowpea and lupine nodule bacteria tested is not related to their ability to cross-inoculate.

The results of the many agglutination tests in which various antigenic modifications of 54 strains of soybean, cowpea and lupine rhizobia were tested against the 25 different antisera are not presented. There are three reasons for omitting a detailed presentation of these data. In the first place, there is insufficient space for such a mass of tabulated information; secondly, whole-cell antigens used in agglutination tests with antisera prepared with whole cells gave in general the same reactions as did the various antigenic modifications; and finally, the titers secured with whole-cell antigens and whole-cell antisera were as a rule higher than those secured with the other antigenic modifications and their antisera.

However, these tests showed that strains belonging to the same serological type possessed similar antigenic constituents. This is illustrated in the following selected data, which show the highest titer in which agglutination occurred when the various antigenic modifications of certain strains were tested with antisera which would cause their agglutination.

ANTIGENS	ANTISERA EMPLOYED IN TESTS	WHOLE	HEATED	WASHED-HEATED
Cowpea 606 whole	Cowpea 601	6400	2000	800
Cowpea 606 heated	Cowpea 601	2000	800	200
Cowpea 606 washed-heated	Cowpea 601	800	400	200
Soy 505 whole	Soy 505	6400	400	400
Soy 505 heated	Soy 505	1600	400	400
Soy 505 washed-heated	Soy 505	400	800	200
Lupine 807 whole	Soy 505	1600	200	50
Lupine 807 heated	Soy 505	400	100	100
Lupine 807 washed-heated	Soy 505	100	100	100
Soy 508 whole	Lupine 804	6400	1600	400
Cowpea 605 whole	Soy 505	6400	1600	200
Soy 507 whole	Lupine 804	2000	400	100

This diminution of activity, both in agglutinability and in ability to stimulate antibody formation in the animal, strongly suggests the existence of three components in the antigenic complex of these root-nodule bacteria. In the cells that are relatively free from flagella and capsules is an antigen which stimulates the production of agglutinins. Its ability both to stimulate the production of these antibodies and to react with them is very limited, indicating that this antigenic fraction may be present in only very small amounts, or that the treatment the washed-heated antigens received may have denatured it to a considerable extent. The second antigenic substance is apparently associated with the flagella, since depriving the cells of their flagellar proteins by exposing them to heat lowers their ability to induce formation of agglutinins and to react with these antibodies to the degree that is possessed by untreated cells. Finally, there is a fraction, possessed by untreated cells, which may be associated with the polysaccharide capsular gum of the root-nodule bacteria.

It is disconcerting to note, however, that these antigenic constituents, even those of the washed-heated cells, are not found in all strains of a species; nor are they found in all strains that will cross-inoculate in a similar manner.

It is significant that there was no agglutination of the whole-cell test-antigens prepared from mixed strain cultures of alfalfa, clover, and pea rhizobia by the different antisera used in these tests. Apparently these rhizobia differ in their antigenic constitution from any of the soybean, cowpea or lupine strains that were used in these tests.

CONCLUSIONS

1. A great number of serological types exist among the root-nodule bacteria from plants of the soybean, cowpea, and lupine cross-inoculation groups.

2. The strains within a serological type possess antigenic constituents in common. These antigens are found in whole cells, heated cells and in washed-heated cells.

3. There seems to be no correlation between the ability of

strains of rhizobia from soybeans, cowpeas and lupines to cross-inoculate and to cross-agglutinate. One serological type may be made up of *Rhizobium* strains isolated from only one species of plant, but in other cases, of strains isolated from two or even three species of plants.

4. Antisera for the different antigenic modifications of soybean, cowpea and lupine bacteria failed to agglutinate whole-cell antigens of alfalfa, clover or pea bacteria.

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CHEMICAL FACTORS INFLUENCING THE GROWTH OF TUBERCLE BACILLI

II. Organic Reagents

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INTRODUCTION

In a previous paper (Sher and Sweany, 1939) we reported the effects of metal ion catalysts on the growth of tubercle bacilli. When iron salts were added to a Long's synthetic medium which had been prepared from "highly purified reagents," the yields of bacilli were increased; this was especially true if copper, manganese or pyo-antimonate ions were added along with the iron. On the other hand, the non-ferrous catalysts, in the absence of iron, did not yield enhanced growths. As contrasted to this, Long's synthetic media prepared from "C. P." or "Reagent" grade chemicals did not require the addition of iron salts or other substances to yield abundant growths, and their addition only slightly increased the yield of bacilli. This is an indication that there were traces of metals or other impurities in these reagents which acted similarly to the added metal catalysts.

We felt that since these added metal catalysts which caused enhanced growths of tubercle bacilli are also known to accelerate the rates of oxidation of biological and simple thiol systems, that it would be of interest to study the effect of a reagent, such as diphenylamine, which is known to act as an inhibitor for oxidation. Kharasch, *et al.* (Kharasch, Conway, and Bloom, 1936) found previously that diphenylamine profoundly influenced the growth of various bacteria on different media. This remarkable effect is in accord with the results obtained in our laboratory with

tubercle bacilli: diphenylamine inhibited the growth of tubercle bacilli in all of the media used.

PREPARATION OF MATERIALS

The media and equipment were prepared with the precautions described in our previous report (Sher and Sweany, 1939).

The amounts of inhibitors incorporated into the media were so small that it was impractical to weigh the amount required for each concentration; therefore, the quantities necessary for the greatest concentrations were weighed on a micro-analytical balance and were then incorporated into the correct amount of liquified medium by means of vigorous agitation. For each subsequent dilution, two parts of liquified medium were mixed with one part of the more concentrated medium.

Since some of the organic inhibitors used in this investigation were but slightly soluble in the media used, they were probably present as an emulsion rather than as a solution. Because of this factor great care must be exercised if reproducible results are to be obtained.

EXPERIMENTAL RESULTS

In order that we might more readily familiarize ourselves with the effects of diphenylamine and other organic inhibitors we repeated and extended some of the work of Kharasch and others, with *Staphylococcus aureus* and ran concurrent comparative studies with bovine tubercle bacilli (B599). These preliminary studies were carried out with non-synthetic media and are presented in tables 1, 2, and 3. Table 4 contains the results of table 3 and data obtained on *synthetic* media with bovine (B599) and human (H37) strains of tubercle bacilli.

Table 1 indicates that the first generation of *Staphylococcus aureus* did not grow on meat broth 2 per cent agar medium which contained diphenylamine in a concentration of 1:2,000. At lower concentrations, depigmented growths were obtained which varied with the concentration of the diphenylamine. After the third generation, the organism had adapted itself to the medium containing diphenylamine to such an extent that unimpaired non-

pigmented growths appeared in a concentration of 1:2,000. In general, only in concentrations between 1:2,000 to 1:6,000 was there a consistent non-pigmented growth. This effect did not

TABLE 1

The effect of diphenylamine on growth and pigmentation of Staphylococcus aureus

DIPHENYLAMINE MEDIA* CONCENTRATION	1ST GENERATION GROWTH AFTER 3RD DAY	2ND GENERATION GROWTH AFTER 3RD DAY	3RD GENERATION GROWTH AFTER 3RD DAY	4TH GENERATION GROWTH AFTER 3RD DAY	5TH GENERATION GROWTH AFTER 3RD DAY	6TH GENERATION GROWTH AFTER 3RD DAY
$\frac{1}{2,000}$	No growth	Poor non-pigmented	Good non-pigmented§	Good non-pigmented¶	Good non-pigmented‖	Good non-pigmented
$\frac{1}{6,000}$	Good non-pigmented†	Good non-pigmented	Good non-pigmented	Good non-pigmented	Good non-pigmented	Good dull orange
$\frac{1}{18,000}$	Good non-pigmented	Good non-pigmented‡	Good pale orange	Good pale orange	Good pale orange	Good dull orange
$\frac{1}{50,000}$	Good dull orange	Good dull orange	Good pale orange	Good dull orange	Good pale orange	Good dull orange
$\frac{1}{150,000}$	Good dull orange	Good dull orange	Good dull orange	Good dull orange	Good dull orange	Good dull orange
$\frac{0}{\text{media}}$	Good dull orange	Good dull orange	Good dull orange			

* Meat broth 2 per cent agar media.
† Growths used to culture the 2nd generation.
‡ Growths used to culture the 3rd generation.
§ Growths used to culture the 4th generation.
¶ Growths used to culture the 5th generation.
‖ Growths used to culture the 6th generation.

persist after these cultures were transplanted on media not containing diphenylamine, for, when transplanted onto such a medium even after growing for five generations as a non-pigmented growth, pigmentation reappeared. Within the scope of

our experiments, succeeding generations of B599 which were grown on similar media did not adapt themselves to media containing successively higher concentrations of diphenylamine.

Table 2 indicates that on meat-broth-agar medium diphenylamine was more effective in retarding growth and in causing a non-pigmented growth than the other organic inhibitors in this

TABLE 2

Effect of organic inhibitors on Staphylococcus aureus and tubercle bacillus (B599) in meat broth 2 per cent agar media

ORGANIC INHIBITOR	STAPHYLOCOCCUS AUREUS	B599
Diphenylamine		
1:2,000	No growth	No growth
1:6,000	Retarded and non-pigmented growth	No growth
1:18,000	Growth but non-pigmented	No growth
1:50,000	Good growth initially non-pigmented	Retarded growth
p-methylamino-phenol sulfate		
1:6,000	Retarded and non-pigmented growth	Retarded growth
1:18,000	Retarded, but normal pigmented growth	Retarded growth
Catechol		
1:2,000	Retarded normal pigmentation	No growth
1:5,000	Normal growth	No growth
1:15,000	Normal growth	Retarded growth
p-hydroxy phenyl glycine		
1:2,000	Normal growth	No growth
1:5,000	Normal growth	No growth
1:50,000	Normal growth	Retarded growth

series. Incidentally, these inhibitors exerted less of an inhibiting effect on the growth of *Staphylococcus aureus* than on B599.

Attempts to grow the strains of *Staphylococcus aureus* which we studied, on Long's synthetic 2-per-cent agar medium failed. But when calves lung extract was added to this medium a much brighter orange growth appeared than when the bacterium was grown on meat broth medium. The lung extract apparently contained a growth essential that is more in the nature of a food requirement than a catalyst, for growth was proportional to the

amount of extract added. When 1 per cent of the lung extract was added, an abundant growth was obtained; with $\frac{1}{2}$ of 1 per cent a fair growth was obtained; with $\frac{1}{6}$ of 1 per cent only a poor growth appeared, while at $\frac{1}{18}$ of 1 per cent there was no growth. Diphenylamine and alpha-naphthylphenylamine inhibited the growths of *S. aureus* and B599 on Long's media containing 1 per cent of the lung extract. Table 3 indicates the effectiveness of diphenylamine as an inhibitor in this medium.

Table 4 indicates that the addition of p-hydroxyphenylmorpholine and trimethylphenyl ammonium hydroxide to the media,

TABLE 3

Effect of inhibitors on "C.P." 2 per cent agar media with 1 per cent lung extract

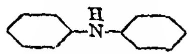
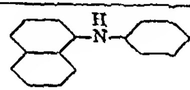
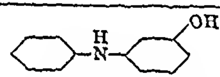
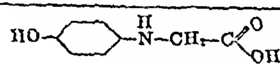
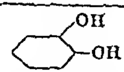
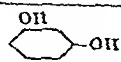
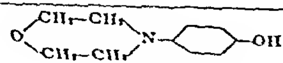
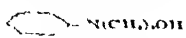
ORGANIC INHIBITOR	STAPHYLOCOCCUS AUREUS	B599
Diphenylamine		
1:30,000	No growth	No growth
1:9,000	Non-pigmented (unimpaired growth)	No growth
1:27,000	Non-pigmented (unimpaired growth)	Retarded
1:81,000	Normal growth	Retarded
α-naphthylphenyl amine		
1:6,000	Non-pigmented (unimpaired growth)	Retarded
1:18,000	Normal growth	Normal growth

instead of inhibiting, slightly increased the rates and amounts of growth of tubercle bacilli.

Table 4 also contains the results of a large number of experiments using a number of inhibitors on a bovine (B599) and a human strain (H37) of tubercle bacilli, employing ordinary "C. P." as well as "highly purified media." Our data are not sufficiently extensive to draw far-reaching conclusions, yet they indicate that on "highly purified media" the bacteria are much more sensitive to the action of the inhibitors than on the media containing ordinary "C. P." reagents. In some cases the results are most striking, for a ten-fold difference in effect can be observed in the case of catechol. Thus, on a 2-per-cent "C. P." agar medium

TABLE 4

Effects of inhibitors on growth of tubercle bacilli and Staphylococcus aureus

			1	2	3	4	5	6
MICROORGANISM			B599	B599	B599	H37	H37	*Staphylococcus aureus*
MEDIA			"CP" 2 per cent agar	Purified 2 per cent agar	Meat broth 2 per cent agar	Purified 2 per cent agar	Purified 2 per cent agar	Meat broth 2 per cent agar
INHIBITOR	STRUCTURE	EFFECT ON GROWTH						
Diphenylamine		Prevented Retarded	1:15,000 1:50,000	1:15,000 1:50,000	1:18,000 1:50,000	1:50,000 1:50,000	1:50,000 1:50,000	1:2,000 1:18,000
a-Naphthyl phenylamine		Prevented Retarded	1:15,000 1:50,000	1:15,000 1:50,000		1:50,000 1:50,000	1:50,000 1:50,000	
m-Hydroxydi-phenylamine		Prevented Retarded	1:5,000 1:15,000	1:5,000 1:15,000		1:50,000 1:50,000	1:50,000 1:50,000	
p-Methylamino phenolsulfate		Prevented Retarded	1:15,000 1:50,000	1:50,000 1:50,000	1:6,000 1:18,000	1:5,000 1:15,000	1:5,000 1:15,000	1:6,000 1:15,000
p-Hydroxy phenylglycine		Prevented Retarded	1:5,000 1:15,000	1:15,000 1:50,000	1:5,000 1:50,000	1:5,000 1:15,000	1:15,000 1:50,000	1:2,000 1:12,000
Catechol		Prevented Retarded	1:5,000 1:15,000	1:50,000 1:50,000	1:5,000 1:15,000	1:15,000 1:50,000	1:15,000 1:50,000	1:2,000 1:2,000
Hydroquinone		Prevented Retarded	1:5,000 1:15,000	1:15,000 1:50,000		1:5,000 1:15,000	1:5,000 1:50,000	
Resorcinol		Prevented Retarded	1:5,000 1:15,000	1:15,000 1:50,000		1:5,000 1:15,000		
p-Hydroxy phenyl morpholine		Enhanced	1:5,000	1:5,000		1:5,000		
Trimethyl phenyl [illegible]		Enhanced	1:5,000			1:5,000	1:5,000	

it prevents growth in 1:5000, and on the "highly purified media" it prevented growth in 1:50,000. These results were so outstanding that they were duplicated many times in our laboratory. In other cases the results were less outstanding, yet the trend was unmistakable. Thus, instead of a ten-fold effect, only a three-fold effect was observed with a number of the other hydroxy compounds. Strikingly, the diphenylamine series of compounds shows no difference in inhibiting power in "highly purified" and normal media. These results are highly suggestive, but it is fully realized that further data are needed to enable us to evaluate the factors responsible for the results.

We believe that it is of considerable interest that these antioxidants have a tremendous inhibitory power on bovine, and in particular, on human strains of tubercle bacilli. The tremendous inhibitory effect observed for instance, on H37, the human strain of tubercle bacilli, in which growth is prevented in 1:50,000 by the presence of diphenylamine, as compared to 1:2000 necessary to prevent growth of *Staphylococcus aureus* is highly suggestive.

We intend to continue this study of the relationship of the antioxidants and the oxidation-reduction potential, or in some cases, as what is commonly known as "apparent" oxidation-reduction potential, of these antioxidants in relation to their effect on the growth of tubercle bacilli.

SUMMARY

1. The diaryl secondary amines investigated were uniform in their activity as inhibitors regardless of whether "C. P.", "Purified" or non-synthetic medium was used.

2. Each of the dihydroxybenzenes and the aryl-aliphatic secondary amines was more effective in "Purified" medium than in "C. P." or meat broth medium.

3. Some of the aryl-aliphatic secondary amines and dihydroxybenzenes were more effective than the diaryl secondary amines in "Purified" medium. In the "C. P." or meat broth medium they were less effective.

4. A number of antioxidants were tested for their inhibiting effect on the growth of tubercle bacilli. No attempt has been

made at this time to correlate the oxidation-reduction potential, or "apparent" oxidation-reduction potential of these materials and their effectiveness in inhibiting the growth of tubercle bacilli. This work is being continued.

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THE LACK OF ONE OF THE SOMATIC ANTIGENS OF TYPHOID CULTURES

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Naturally occurring, smooth, motile cultures of *Eberthella typhosa* contain, according to the best of present-day knowledge, a heat-labile, specific flagellar, or "H" antigen, called "d" in the Kauffmann scheme of classification; two heat-stable, somatic, or "O" antigens, designated as "IX" and "XII"; and a heat-labile, somatic antigen, the "Vi" of Felix and Pitt, 1934, called "A" in the Kauffmann scheme. In addition an alternate "H" antigen, "j," has been demonstrated by Kauffmann, (1936), in a very limited number of specially treated cultures, but has never been found in cultures isolated by the usual methods.

Variations involving loss of the "H" factor and loss of the "Vi" factor without alteration of the smooth character of the cultures or of the fermentation characteristics have been commonly reported. Those variations which involve the "O" antigens, however, have usually been described in connection with the smooth to rough transition.

The evidence here detailed indicates the lack in two cultures of the typhoid organism of one of the heat-stable somatic factors without any easily demonstrable change in cultural characteristics.

EVIDENCE FROM THE ACTION OF BACTERIOPHAGE

The cultures of *Eberthella typhosa* in stock, numbering 170, were tested for their susceptibility to a series of eight bacteriophages, active also against various members of the *Salmonella* group. The method employed was that described by Craigie, 1936, in connection with his work on V phage. It consists in

inoculating the surface of a plain agar plate with a four-hour broth culture of the organism tested and placing a loopful of undiluted bacteriophage upon the inoculated area. Observations are made after about twelve hours for the appearance of the area where the phage has been applied. The criterion for complete sensitivity is the absence of any colonies within this area after

TABLE 1

The action of bacteriophages from various sources on typhoid cultures

	PHAGES								
	PA 4*	PB 15	62	88	92	L	S	K	V
T 2	ACC	NI	PC	ANI	ACC	PC	ANI	ANI	CC
T 48	ACC	NI	PC	ANI	ACC	PC	ANI	ANI	CC
T 4	CC	CC	CC	CC	CC	CC	CC	CC	CC
H 901	CC	CC	CC	CC	CC	CC	CC	CC	NI
Para B 13	CC	CC	PC	PC	ANI	PC	PC	PC	NI
Enteritidis 34	ACC	ACC	CC	CC	ACC	ACC	ACC	ACC	NI

Symbols describing areas where phage was applied: CC, completely clear; ACC, almost completely clear; i.e., a few isolated colonies; PC, partially clear; i.e., numerous isolated colonies; ANI, almost no inhibition of growth; i.e., colonies confluent; NI, no inhibition of growth; i.e., no visible difference between area where phage was applied and surrounding inoculated area.

* The first five phages in the table were obtained from A. W. Frisch.

TABLE 2

The absorption of phage PB 15 by three typhoid cultures

Test organism, T 4

ABSORBING CULTURES	PHAGE DILUTIONS					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
T 2	Cl	Cl	Cl	Cl	±	+++
T 48	Cl	Cl	±	±	+	+++
T 4	+	+++	+++	+++	+++	+++
Unabsorbed control	Cl	Cl	Cl	Cl	Cl	+++

the stated incubation period. Nearly all the cultures thus tested showed complete or good sensitivity to the eight phages of various origins. This is in accordance with the statement of Kauffmann, (1936), that phages active against typhoid organisms are of two kinds only—those which act on the V form alone and on few or no *Salmonella* types, and those which act on V and W forms of

the typhoid organism indiscriminately and on numerous *Salmonella* species as well. The eight phages employed in our experiment were of the type which acted on both V and W forms and on numerous *Salmonella* species. (Our work with the V phage of Craigie has been previously reported; Almon and Stovall, 1936). That these eight phages were not all alike in their action on the typhoid cultures, however, was shown by the complete failure of one of them to act on two of the cultures investigated, and by the weak action of three others on the same cultures. Table 1 is a section from the protocols of this experiment. It shows the action of the eight phages just mentioned, as well as that of the V phage of Craigie, on six cultures. Cultures T 4 and H 901 are included as representatives of the large number which were completely sensitive to the eight *Salmonella* phages. Cultures "para B 13" and "enteritidis 34" are included also, because they were used in the subsequent serological work. Cultures T 2 and T 48, quite evidently, are the two resistant typhoid strains.

The demonstration of the specificity of phage activity has been shown by Frisch and Levine, 1936, to be better carried out by absorption technique than by observation of direct lysis. Therefore, in order to verify the seeming difference between the typhoid cultures sensitive to all the phages and those two which were resistant or partially resistant to several, an absorption experiment was carried out. The technique was that of Levine and Frisch, 1935, in all particulars. The phage used in the experiment was PB 15, the one which had failed entirely to act on cultures T 2 and T 48 in the previous experiment. Absorptions were carried out with cultures T 2, T 48, and T 4; and tests for residual active phage were carried out with T 4 as the sensitive organism. The readings reported in table 2 were taken twenty hours after the broth and inoculum of sensitive organisms had been added to the incubated mixture of phage dilution and absorbing organisms. The symbol "Cl" denotes clearing and indicates lack of absorption of the phage dilutions so described. It is readily apparent, therefore, that cultures T 2 and T 48 were poor absorbing agents as compared with culture T 4.

Investigation of the difference revealed by these results might proceed along two lines; either along the line of analysis of phage specificity or along the line of serological analysis of the cultures. Since the first mentioned is dependent upon the second, the latter, or serological approach, was selected.

The most likely explanation of the differences observed in cultures T 2 and T 48 as compared with the other typhoid cultures was thought to be the absence from these two cultures of some fraction present in other typhoid cultures. Since the work of Levine and Frisch, 1935, has shown that there is close correlation between sensitivity to specific phages and the presence of certain heat-stable "O" components among members of the *Salmonella* group, it seemed probable that in these cases also an "O" component was involved. It could, indeed, scarcely be anything but an "O" component, since the two cultures were actively motile, and thus apparently possessed of the "H" antigen, and since previous (Almon and Stovall, 1936) and present tests had demonstrated the presence of the "Vi" antigen.

SEROLOGICAL EVIDENCE

The possibility of the lack of an antigenic fraction was investigated by the absorption of agglutinins. Because an "O" component seemed most probably involved, the methods chosen were those best adapted to the demonstration of "O" antibodies.

Immunization of rabbits was carried out with suspensions killed by heating at 65°C. for one hour. Five intravenous injections, given at two day intervals, were sufficient to bring the titer to the desired level. The blood was drawn eight days after the last injection. Heat-killed cultures were used also for the agglutination tests, both before and after the absorption of the sera. For the absorbing doses, however, live organisms were used, in order that the "H" antibodies (which are formed to some extent even when the immunizing cultures are killed by heat) might be removed. The 24-hour growth from five plain agar plates constituted a single absorbing dose for 10 ml. of a 1:20 dilution of antiserum, but two successive doses of this kind were employed for complete absorption.

TABLE 3

The results of agglutinin absorption tests on antisera for two unusual and two usual typhoid cultures

	ANTIGENIC SUSPENSIONS, HEAT KILLED			
	T 2	T 48	T 4	H 901
	Titers			
Antiserum T 2:				
Before immunization	0*	0	1:160	1:40
After immunization, unabsorbed	1:10240	1:10240	1:10240	1:10240
After immunization, absorbed by T 2..	0	0	0	0
After immunization, absorbed by T 48..	0	0	0	0
After immunization, absorbed by T 4..	0	0	0	0
After immunization, absorbed by H 901.	0	0	0	0
Antiserum T 48:				
Before immunization	0	0	1:1280	1:320
After immunization, unabsorbed.	1:10240	1:5120	1:10240	1:10240
After immunization, absorbed by T 48..	0	0	0	0
After immunization, absorbed by T 2..	0	0	0	0
After immunization, absorbed by T 4..	0	0	0	0
After immunization, absorbed by H 901.	0	0	0	0
Antiserum T 4:				
Before immunization	0	0	0	0
After immunization, unabsorbed.	1:10240	1:10240	1:10240	1:10240
After immunization, absorbed by T 4..	0	0	0	0
After immunization, absorbed by H 901.	0	0	0	0
After immunization, absorbed by T 2	0	0	1:1280	1:1280
After immunization, absorbed by T 48..	0	0	1:2560	1:2560
Antiserum H 901:				
Before immunization	0	0	0	0
After immunization, unabsorbed.	1:1280	1:1280	1:10240	1:10240
After immunization, absorbed by H 901.	0	0	0	0
After immunization, absorbed by T 4..	0	0	0	0
After immunization, absorbed by T 2..	0	0	1:2560	1:2560
After immunization, absorbed by T 48..	0	0	1:1280	1:1280

* Titers reported as "0" really mean "less than 1:40," since no tests were carried out with dilutions lower than this figure.

The organisms chosen as representative of the usual type of typhoid cultures were T 4 and H 901. The latter was included because it contains no Vi antigen to confuse the results; though

the elimination of confusion by this factor was further brought about by the use of heat-killed suspensions.

The outcome, set forth in table 3, provided additional evidence for the absence from cultures T 2 and T 48 of an antigenic component which is present in T 4 and H 901; the latter two cultures were able completely to absorb antisera resulting from immunization by the former; but absorption by the former cultures, the seemingly deficient ones, left antibodies in the T 4 and H 901 antisera for the homologous cultures. Henceforward in this paper, cultures T 2 and T 48 will be spoken of as "deficient" cultures and the other typhoid cultures, of which T 4 and H 901 have been used as representatives, will be called "complete."

IDENTIFICATION OF THE MISSING COMPONENT

Whether the seeming lack of an "O" component were due to the absence of one or the other of the two assigned by the Kauffmann classification or whether a hitherto unidentified component were involved could not be determined from the evidence at hand. The specificity of the phages employed had not been investigated sufficiently to afford any clue. It was known, however, that phage PB 15, to which the cultures in question were most completely resistant, had originally been propagated on a culture of *Salmonella schottmuelleri*, albeit a rough one. It was assumed, therefore, that the component which was lacking was the one responsible for cross reactions with the paratyphoid B group, namely antigen XII (Kauffmann, 1935b). Experiments were undertaken to investigate the validity of this hypothesis.

This investigation necessitated the immunization of a rabbit with a culture of *Salmonella schottmuelleri*, and the absorption of the resulting antiserum by the two types of typhoid cultures, as well as the absorption of the previously prepared typhoid antisera by the *S. schottmuelleri* culture. The culture used was the "para B 13" from the Rockefeller Institute, supplied to us by Dr. P. Levine. The methods of immunization and absorption were identical with those described in the foregoing section. Care was taken to select for immunization with the para B culture

a rabbit which had no demonstrable normal antibodies for any of the cultures used in this work. A preliminary experiment had shown that the presence of such antibodies so complicated the results that they were difficult to interpret. Table 4 represents the protocols of the cross absorptions with the para B culture. Absorptions of the typhoid sera by the typhoid cultures were presented in table 3 and are not repeated here, though the results are pertinent in this connection also.

The evidence given by these results is preponderantly in favor of identity of the "O" fraction remaining in the deficient cultures with that component which *Salmonella schottmuelleri* shares with *Eberthella typhosa,* rather than in favor of the proposed hypothesis which led to this experiment. This is best shown by the ability of the deficient and the complete typhoid cultures, alike, completely to remove the typhoid antibodies from the paratyphoid antiserum, and of all of the typhoid cultures to remove the paratyphoid antibodies from the typhoid sera. Additional evidence in support of this conclusion comes from the demonstrated ability of the paratyphoid culture to remove all of the antibodies from the antisera prepared against one of the complete cultures; *vide* absorption of H 901 antiserum by para B 13. That the para B culture did not completely remove the antibodies for the deficient cultures from the other typhoid antisera is the only evidence not completely in accord with the theory of the identity of the "O" component of T 2 and T 48 with antigen XII, the one shared by *E. typhosa* and *S. schottmuelleri.* Taking into consideration the manifestly poor antigenic qualities of the *S. schottmuelleri* culture with respect to this component, as evidenced by the low titer of its antiserum for the typhoid cultures, this failure may not necessarily be at variance with the behavior expected to conform with the theory just outlined—that of identity of the "O" component in the deficient typhoid cultures with antigen XII, shared by *S. schottmuelleri.* In brief, the para B 13 culture is evidently a poor absorbing agent for typhoid antibodies, and doses of sufficient magnitude to remove these antibodies from a serum of high titer are impractical to work with. The significant thing is that in the one antiserum (H 901) in

TABLE 4

Cross agglutinations and absorptions with typhoid cultures and a paratyphoid B culture

	PARA B 13	T 2	T 48	T 4	H 901
	Titers				
Antiserum Para B 13:					
Before immunization	0	0	0	0	0
After immunization, unabsorbed	1:10240	1:80	1:160	1:320	1:320
After immunization, absorbed by Para B 13 ..	0	0	0	0	0
After immunization, absorbed by T 2	1:5120	0	0	0	0
After immunization, absorbed by T 48	1:5120	0	0	0	0
After immunization, absorbed by T 4	1:5120	0	0	0	0
After immunization, absorbed by H 901 .	1:5120	0	0	0	0
Antiserum T 2:					
After immunization, unabsorbed	1:160	1:10240	1:10240	1:10240	1:10240
After immunization, absorbed by Para B 13	0	1:2560	1:2560	1:2560	1:2560
After immunization, absorbed by T 2	0	0	0	0	0
After immunization, absorbed by H 901 . .	0	0	0	0	0
Antiserum T 48:					
After immunization, unabsorbed	1:1280	1:10240	1:5120	1:10240	1:10240
After immunization, absorbed by Para B 13	0	1:2560	1:2560	1:2560	1:2560
After immunization, absorbed by T 2	0	0	0	0	0
After immunization, absorbed by H 901	0	0	0	0	0
Antiserum T 4:					
After immunization, unabsorbed	1:320	1:10240	1:10240	1:10240	1:10240
After immunization, absorbed by Para B 13	0	1:2560	1:2560	1:2560	1:2560
After immunization, absorbed by T 2	0	0	0	1:1280	1:1280
After immunization, absorbed by H 901	0	0	0	0	0
Antiserum H 901:					
After immunization, unabsorbed	1:320	1:1280	1:1280	1:10240	1:10240
After immunization, absorbed by Para B 13	0	0	0	1:640	1:1280
After immunization, absorbed by T 2	0	0	0	1:2560	1:2560
After immunization, absorbed by H 901 .	0	0	0	0	0

which antibodies for the deficient cultures were present in lower titer, the para B culture was able to remove them completely.

The evidence is therefore reasonably good that the deficient cultures contain antigen XII of the Kauffmann scheme. To prove that they lack the other known "O" component, namely antigen IX, is virtually impossible with the reagents and knowledge at present available, for there is no species of organism known which contains antigen IX and lacks antigen XII. Hence we have been unable to prepare an antiserum containing antibodies for factor IX alone of the somatic typhoid group and thus to demonstrate the inability of cultures T 2 and T 48 to be agglutinated by this antibody. The nearest we could approach the identification of the seemingly absent component was to attempt the demonstration of whether a component hitherto unidentified were involved, or whether it were one which is gen-

TABLE 5

Cross agglutinations and absorptions with typhoid cultures and a culture of Salmonella enteritidis

	ENT. 34	T 2	T 48	T 4	H 901	PARA B 13
Antiserum Enteritidis 34:						
Before immunization	0	1:40	1:40	1:40	0	1:40
After immunization, unabsorbed	1:5120	1:5120	1:5120	1:5120	1:5120	1:1280
After immunization, absorbed by Ent. 34	0	0	0	0	0	0
After immunization, absorbed by T 2	1:160	0	0	1:160	1:160	0
After immunization, absorbed by T 4	0	0	0	0	0	0
Antiserum T 2:						
After immunization, unabsorbed	1:2560	1:10240	1:10240	1:10240	1:10240	1:160
After immunization, absorbed by Ent. 34	0	0	0	0	0	0
Antiserum H 901:						
After immunization, unabsorbed	1:2560	1:1280	1:1280	1:10240	1:10240	1:320
After immunization, absorbed by Ent. 34	0	0	0	0	0	0
After immunization, absorbed by T 2	1:320	0	0	1:640	1:640	0

erally conceded to be part of the typhoid organism. This was carried out by cross-absorption experiments with a culture of *Salmonella enteritidis* (number 34 of Levine and Frisch, 1935) the results of which appear in table 5. The T 2 antiserum is included as the representative of the deficient sera and H 901 as representative of the complete ones. Results with the two sera omitted from the table (T 48 and T 4) were similar. It may be seen from a study of these results that the antiserum prepared against the heat killed *S. enteritidis* culture presented an analyzed antibody content in all respects like antisera for the somatically complete typhoid cultures, in that its antiserum could be completely absorbed of antibodies for both typhoid and enteritidis by the complete typhoid cultures. Similarly, the enteritidis culture behaved in its absorbing capacity like a typhoid culture with full complement of somatic antigens, since it was able to absorb all of the antibodies from the typhoid antisera of either the complete or the deficient type. More significant in its bearing on the present arguments was the observation that the deficient cultures, in their absorption of the *S. enteritidis* antiserum, left antibodies to an equal titer for the complete typhoid cultures and for the enteritidis culture. All of these results point to the conclusion that the difference between the deficient and complete typhoid cultures lies simply in the absence from the deficient cultures of a somatic fraction present in other typhoid cultures and in *S. enteritidis*. Since, according to the evidence derived from cross-absorption tests with *S. schottmuelleri*, the deficient cultures possess antigen XII, and since, according to cross absorption tests with *S. enteritidis*, the missing component is present in this species, it may be concluded that the fraction which is absent is antigen IX of the Kauffmann scheme.

DISCUSSION

The above conclusion is in line with the report of Kauffmann (1935a) concerning the lack of antigen V in certain cultures of the paratyphoid B group. In his cultures, too, no abnormality

in cultural or pathogenic characteristics was noted. In this connection it may be of interest that culture T 2 was isolated from the blood of a fatal case of typhoid fever, and that culture T 48 was derived from the feces of a typhoid carrier. We have no history of the cases traced to this carrier.

That the seeming deficiency herein described may be quantitative rather than qualitative is conceded. It is possible that the future will reveal the existence of a number of antigenic strains within the various species of *Salmonella* and *Eberthella* comparable in number and complexity with those already demonstrated for the Flexner dysentery organism. For example, in the course of this work two cultures of *S. schottmuelleri* were found to be unable to produce antibodies in rabbits for *E. typhosa*, evidence that in this species also there may be variations in somatic antigenic content without accompanying variations in cultural characteristics.

More complete knowledge concerning these variations may make for more accurate identification of recently isolated cultures belonging to these groups, and may in some instances facilitate epidemiologic investigations.

SUMMARY

Among 170 cultures of *Eberthella typhosa* two showed resistance to the action of several bacteriophages which were lytic for the others. A phage-absorption experiment, using these two cultures, indicated that the difference was more than superficial; and serologic agglutination studies revealed a deficiency in the somatic make-up of these two cultures.

Cross absorption tests showed that the missing antigen was not the one which *E. typhosa* shares with *Salmonella schottmuelleri*, but was one which is normally present in *S. enteritidis*. On the assumption that the somatic antigenic fractions of *E. typhosa* are limited in number to two (since there was no evidence to the contrary apparent from these studies) the component which was lacking was tentatively identified as antigen IX of the Kauffmann scheme of classification.

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THE GROWTH OF DICTYOSTELIUM DISCOIDEUM UPON PATHOGENIC BACTERIA[1]

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INTRODUCTION

Species of the Dictyosteliaceae, a group of pseudoplasmodium-forming slime molds, are not uncommon in nature and can be readily isolated from soils, from decaying vegetation and from the dung of various animals. In contrast to the *Myxogastrales* which possess true plasmodia, the Dictyosteliaceae are characterized by myxamoebae which retain their identity throughout the whole life cycle of the organisms. During the vegetative stage these myxamoebae are free-living and feed by the ingestion and digestion of bacterial cells. With the exhaustion of the available food supply, the myxamoebae collect into aggregates termed pseudoplasmodia, and collectively build fruiting structures, or sorocarps. In these structures some myxamoebae become transformed into sterile, supportive cells to form a stalk, or sorophore, while others become differentiated into fertile spores forming a spore head, or sorus.

The ubiquitous species *Dictyostelium mucoroides* Bref. has been more widely studied than other members of the group and from the work of Vuillemin (1902), Potts (1902), Pinoy (1903 and 1907), and Schuckmann (1924) a limited bacterial "host range" embracing twelve different species has been reported for it.

[1] Contribution from the Laboratories of Cryptogamic Botany and the Farlow Herbarium, Harvard University, No. 171. A portion of the results here reported were included in a thesis submitted by the senior author to the faculty of Harvard University, June 1936, in partial fulfillment of the requirements for the degree of doctor of philosophy in biology.

Recently *Dictyostelium discoideum* Raper, has been cultivated in pure-mixed culture with thirty-one different species of saprophytic bacteria representing widely separated groups (1937). This study enlarged by almost threefold the number of bacteria with which a species of the Dictyosteliaceae is known to be able to grow.

In the present investigation the writers have studied the growth of *D. discoideum* in association with certain bacteria that are pathogenic to animals or to plants. Such a study seemed particularly desirable in view of the limited attention earlier investigators had given to the cultivation of related slime molds upon bacteria pathogenic to animals. The present work reports for the first time the cultivation of a species of the Dictyosteliaceae with phytopathogenic bacteria.

MATERIALS AND METHODS

D. discoideum was chosen for this study above other species of the Dictyosteliaceae because of two significant characteristics. First, due to the peculiar migrating habit (Pl. 1, fig. 6b) of its pseudoplasmodia (Raper, 1935) spores entirely free from bacteria are regularly borne on sorocarps that develop 0.5 cm. or more distant from the margin of the bacterial colony (Pl. 2, fig. 1c) in which the myxamoebae grew and in which the pseudoplasmodia formed (Raper, 1937). The importance of this character is at once apparent as it permits the investigator to inoculate this slime mold in pure-mixed culture with any selected bacterial culture at will by selecting isolated sorocarps as spore sources. Second, one is able to compare quantitatively the growth of this slime mold upon different bacterial species with a degree of accuracy that is impossible with other species of the group. This is true because the mature fructifications of *D. discoideum* are regularly erect and can be counted (Pl. 2, fig. 1b) whereas those of other species are commonly decumbent, long, and tangled to such an extent as to preclude their enumeration (Pl. 2, fig. 5a). Furthermore, in *D. discoideum* the proportion of stalk mass to spore mass remains fairly constant in all normal sorocarps irrespective of their size, thus enabling one to express conveniently

the growth represented by sorocarps of different size in terms of common units, such as medium sorocarps or "medium-sorocarp equivalents",[2] by computing the volumes of sori of different sized sorocarps.

Dictyostelium discoideum was originally isolated from leaf mould (Raper, 1935), and other species of the Dictyosteliaceae such as *Dictyostelium mucoroides*, *Dictyostelium purpureum* Olive and *Polysphondylium violaceum* Bref. regularly occur in decaying vegetation and in soil (Raper and Thom, 1932). Accordingly for this study there have been selected chiefly species of pathogenic bacteria which under certain conditions may occur in soil.

Hay infusion agar (Raper, 1937) was employed for all cultures where quantitative measurements and comparisons of growth with different bacterial species were contemplated. It was chosen for two reasons: First, it had already been successfully used for cultivating *D. discoideum* in association with a large number of saprophytic bacteria (Raper, 1937); and second, its use in the present study made possible a quantitative comparison of the growth of this slime mold upon pathogenic bacteria with its earlier growth upon non-pathogenic bacteria.

As in the earlier study (Raper, 1937), each of the selected bacterial cultures was inoculated in triplicate upon hay-infusion agar plates in the following manner: Six colonies were established at regular intervals near the periphery of a plate and a single colony at its center. For each colony the inoculum was spread over an area of approximately 1 cm^2. The cultures of plant pathogens were incubated at 22 to 24°C. throughout the experiments, whereas the cultures of animal and human pathogens were incubated at 26 to 28°C. prior to inoculation with *Dictyostelium* and at 20 to 22°C. after the introduction of the slime mold. In all cases the spores of *D. discoideum* were planted after the bacteria had grown for 2 to 3 days. The spores used for inoculation

[2] This unit was first introduced for quantitatively comparing the growth of *D. discoideum* in association with different saprophytic bacteria (Raper, 1937): a detailed account of the procedure employed in effecting the necessary conversions was given at that time.

were invariably obtained from sorocarps that had developed 0.5 cm. or more beyond any evidence of bacterial growth (Pl. 2, fig. 1c), the purity of the inoculum being repeatedly verified by streaking spores from similarly located sorocarps upon nutrient agar. Spores were planted in each of the colonies in two plates and in six of the seven in the remaining plate.

The subsequent growth of *D. discoideum* was determined by counting the number of sorocarps of large, medium and small size in each colony; and from these counts the average growth per colony was obtained for each bacterial culture. To facilitate comparison of the growth of the slime mold with the different bacteria, the average number of large, medium, and small sorocarps per colony was computed in terms of medium sorocarps (Raper, 1937) and expressed as medium-sorocarp equivalents (fig. 1).

EXPERIMENTAL

The results of this study can best be considered by discussing separately the growth of *D. discoideum* in association with bacteria that are pathogenic to plants, to animals, and to man.

With the bacteria pathogenic to plants the slime mold grew well and produced fruiting structures of normal pattern (Pl. 2, fig. 1) in cultures of *Erwinia carotovora*, *Erwinia amylovora*, *Phytomonas phaseoli*, and *Phytomonas flaccumfaciens*. In each case the bacterial colonies were completely consumed by the feeding myxamoebae of the slime mold. Somewhat less growth of the *Dictyostelium* occurred in cultures with *Phytomonas malvacearum*, *Phytomonas medicagenis*, *Phytomonas syringae*, *Phytomonas campestris*, and *Phytomonas phaseoli* var. *fuscus* (fig. 1). In the first three of these cultures the colonies were completely consumed, in the remaining two only partially. Thus the reduced growth of the slime mold in the former cases can be attributed directly to a food shortage; whereas in the latter cases it must be ascribed to the inability of the myxamoebae to feed effectively upon the bacteria present. Such a condition is illustrated even better perhaps by the cultures with *Phytomonas tumefaciens* where good growth of *D. discoideum* occurred in

association with a strain, no. 176, isolated from "crown gall" of peach, while only fair growth of the slime mold took place with a second strain, no. 185, isolated from "hairy-root" of nursery stock (fig. 1). No explanation for this difference in growth of

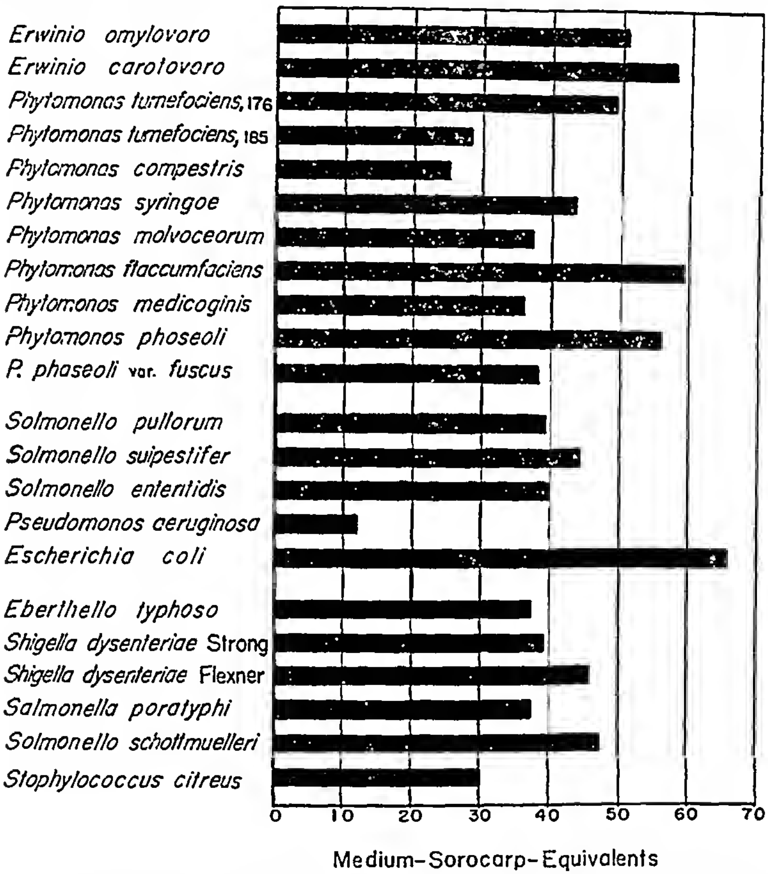

FIG. 1. COMPARATIVE GROWTH UPON HAY INFUSION AGAR OF DICTYOSTELIUM DISCOIDEUM IN ASSOCIATION WITH DIFFERENT BACTERIA PATHOGENIC TO PLANTS, TO ANIMALS AND TO MAN

the slime mold is at hand, for the bacterial growth in the two cases was essentially alike in amount and seemingly in character.

With the bacteria pathogenic to animals *Dictyostelium discoideum* grew fairly well in association with *Salmonella pullorum*, *Salmonella suipestifer* and *Salmonella enteritidis* (fig. 1). In

each case the bacterial colonies were completely consumed by the feeding myxamoebae, pseudoplasmodia began forming two days after the introduction of slime mold spores, and sorocarps of normal pattern were subsequently produced. Since the bacterial colonies were completely consumed, it may be noted that the somewhat limited growth of the slime mold with these cultures is attributable to a correspondingly restricted growth of host bacteria. Excellent growth and normal development of *D. discoideum* occurred in association with *Escherichia coli*[3] whereas fair growth but normal development of the slime mold took place with *Pseudomonas aeruginosa*;[3] the bacterial colonies being wholly consumed in the former case but only partially so in the latter.

Moderately good growth of *Dictyostelium discoideum* occurred with the following human pathogens of the colon-typhoid-dysentery group: *Eberthella typhosa, Shigella dysenteriae* "Flexner," *Shigella dysenteriae* "Strong," *Salmonella paratyphi*, and *Salmonella schottmuelleri*. In all cases the bacterial colonies were wholly consumed by the feeding myxamoebae, pseudoplasmodia were formed after 2 to 2½ days, and sorocarps of entirely normal pattern were subsequently produced. In association with *Staphylococcus citreus* fair growth of *D. discoideum* occurred, the bacterial colonies were largely consumed, and sorocarps of essentially normal pattern were produced.

Realizing that hay infusion agar was at best a poor medium for *Eberthella typhosa* and related bacteria, some cultures of *D. discoideum* in association with *E. typhosa* were subsequently made upon media containing 1 per cent each of peptone and lactose or peptone and glucose. Upon these media occurred a rich growth of bacteria which was wholly consumed by the feeding myxamoebae of the slime mold and fruiting structures were subsequently produced. These, however, were predominantly abnormal in pattern thus indicating the presence in the cultures

[3] In recognition of the fact that these species ordinarily behave as saprophytes they were included in the previous study of the growth of *D. discoideum* with saprophytic bacteria (Raper, 1937); nonetheless, their occasional pathogenicity warrants their inclusion in this study also.

of a condition mildly toxic to *D. discoideum* (Raper, 1939). A more favorable medium consisted of a carrot infusion enriched with 0.5 per cent peptone and buffered with equimolar quantities of monobasic potassium phosphate and dibasic sodium phosphate.[4] Upon this medium the growth of *E. typhosa* approached that upon peptone-lactose and peptone glucose media, the colonies were completely consumed by the feeding myxamoebae, and the production of normal sorocarps indicated that the cultures were wholly favorable for the slime mold (Pl. 2, figs. 2 and 3).

Studies were made of the growth of *D. discoideum* upon three additional species of bacteria that are pathogenic to man, namely: *Staphylococcus aureus*, *Bacillus anthracis*, and *Corynebacterium diphtheriae*. Cultures with these bacteria were not grown upon hay-infusion agar and accordingly the growth of the slime mold with them cannot be quantitatively compared with those previously considered. However, an evaluation of the growth of *Dictyostelium* in association with each will be given. *D. discoideum* was grown in association with *S. aureus* upon a medium containing 1 per cent glucose and 0.5 per cent peptone. Moderately good growth of bacteria occurred, the colonies were completely consumed by the slime mold and some fruiting structures were produced. *B. anthracis* was grown upon a medium containing 1 per cent glucose and 0.2 per cent peptone. Fair growth of the slime mold occurred and sorocarps of normal pattern were formed, but the bacterial colonies were only partially consumed. *C. diphtheriae* was grown upon horse serum-peptone agar for two days at 35°C. after which time the resultant colonies were transferred *en masse* to the surface of nonnutrient agar tubes and inoculated with pure spores of *D. discoideum.* Incubation was at 20 to 22°C. following the introduction of the slime mold. In these cultures the *Dictyostelium* grew well, completely consumed the masses of transferred bacteria and subsequently formed fruiting structures.

[4] Carrot-peptone agar: 300 grams fresh carrots boiled for 1 hour in liter of tap water; filtered; filtrate made up to 1 liter; 0.5 per cent peptone and 1.5 per cent agar added; solution made M/100 in KH_2PO_4 and in $Na_2HPO_4 + 12\ H_2O$; sterilized.

DISCUSSION

The present study greatly enlarges the list of pathogenic bacteria in association with which a species of the Dictyosteliaceae is known to be capable of growing. Previously Pinoy (1907) cultivated *D. mucoroides* with *Vibrio cholera*, *Vibrio metchnikori* and *Bacillus friedlanderi* but failed to culture it with *Bacillus anthracis* or *Pseudomonas aeruginosa*. Potts (1902) likewise attempted unsuccessfully to grow *D. mucoroides* with *B. anthracis*, while Vuillemin (1902) failed to cultivate the slime mold with *P. aeruginosa*. In our experiments *D. discoideum* has been grown with each of these bacteria, although it should be noted, with somewhat less success than with many other pathogens (fig. 1). These divergent results are believed to be due more to the different culture media employed than to any fundamental difference between the nutritional requirements of *D. discoideum* and *D. mucoroides*. Parallel cultures of these slime molds indicate the correctness of such an assumption, for in association with the same species of bacteria and upon media of similar composition the growth of the two species of *Dictyostelium* is regularly comparable (Pl. 2, fig. 4).

The average growth of *D. discoideum* in association with the twenty-one cultures of pathogenic bacteria grown upon hay infusion agar and included in this survey was 42 medium-sorocarp equivalents per bacterial colony, or only slightly less than in association with the saprophytic bacteria previously studied where the average was 44 medium-sorocarp equivalents per colony. This meagre difference would seem to substantiate the conclusion earlier set forth (Raper, 1937) that the growth of *D. discoideum* is not particularly favored by any species or group of bacteria as was maintained by Nadson (1899) and Skupienski (1920) who considered *D. mucoroides* symbiotic with *Bacillus fluorescens-liquefaciens*. Any claim of symbiosis between species of the Dictyosteliaceae and the bacteria associated therewith is refuted by the following facts: (1) They will grow equally well in company with a large number of bacterial species possessing widely different morphological and physiological characters. (2) They are associated in nature with a multitude of bacterial

species, a fact that is attested by the diversity of forms accompanying these slime molds when they are isolated in laboratory culture (Raper, 1937, Table 2).

The myxamoebae of these slime molds are considered by the writers to be predators upon the accompanying bacteria. This interpretation is somewhat at variance with that of Pinoy (1907), who considered *D. mucoroides* to be an obligate parasite infecting bacterial colonies and that of Vuillemin (1902), who described the same species as a "bacteriophage" annihilating bacterial colonies. Pinoy's view would seem unjustified since spores will germinate in the total absence of bacteria, and myxamoebae will feed upon dead bacterial cells as well as living ones. Furthermore, one must ascribe to a bacterial colony an unwarranted unity before one is justified in considering it capable of becoming parasitized. Vuillemin's interpretation more nearly represents the true case. He reported the ingestion and digestion of bacterial cells as individuals by the myxamoebae of *D. mucoroides*, but obviously referred to the macroscopic clearance of bacterial colonies when he termed the slime mold an "Acrasiae bacteriophage." And indeed, colonies of bacteria in which myxamoebae are actively feeding do present pictures simulating those of colonies being destroyed by a phage (Pl. 1, figs. 2 and 3). But the agents here responsible for the destruction of bacterial cells are not submicroscopic in size, but easily observable myxamoebae preying upon bacterial cells, engulfing and digesting them.

Sufficient studies have been made to indicate that either *Dictyostelium mucoroides*, *D. purpureum*, or *Polysphondylium violaceum* will grow with as great a number of different bacteria as *D. discoideum*, and with a vigor equalling or exceeding that species. The latter point is fairly well illustrated in Pl. 2, (figs. 4 and 5) where the growth of *D. mucoroides*, *D. purpureum*, and *D. discoideum* in association with *Eberthella typhosa* may be compared.

The question arises, naturally, as to whether any practical significance can rightfully be attributed to the ability of *Dictyostelium discoideum* to feed upon pathogenic bacteria. This slime mold is probably not widely distributed in nature for it has been

isolated only a few times and only in the writers' laboratory. Further, since in so far as known, it can grow only under strict aerobic conditions and cannot grow at a temperature in excess of 28–30°C., its use as a phagocytic agent within the bodies of animals is precluded. On the other hand, because of the ease with which the species can be cultured in the laboratory and because of the ease with which it can at any time be placed in pure-mixed cultures with any selected culture of bacteria, pathogenic and non-pathogenic alike, it offers an excellent tool with which to study many problems pertaining to the nutrition and feeding habits of amoeboid cells.

While *D. discoideum* is not abundant in nature, other species of the Dictyosteliaceae such as *D. mucoroides*, *D. purpureum* and *Polysphondylium violaceum* are widely distributed and may play a more important rôle in the microbiology of the soil than has hitherto been generally suspected. Although bacteria pathogenic to animals are not commonly encountered in the soil, certain forms may and do occasionally occur there. It is suggested that the fact that they do not long remain may in some measure be due to their removal by the myxamoebae of the Dictyosteliaceae and of the Myxogastrales and the true amoebae of the soil. Finally, as one observes the speed and completeness with which *D. discoideum* (or some other species of the group) consumes large colonies of bacteria in a period of two to three days, one can imagine the possibility of employing these organisms to combat certain bacterial diseases of plants where the inhibiting differentials in temperature characteristic of human pathogens do not occur.

SUMMARY

1. *Dictyostelium discoideum* a non-plasmodium-forming slime mold, was grown in pure-mixed culture upon hay-infusion agar with 22 species and strains of bacteria that are pathogenic to plants, to animals, or to man and its growth with these organisms quantitatively compared.

2. Included in this number as bacterial associates were common plant pathogens such as *Erwinia amylovora*, *E. carotovora*, *Phyto-*

monas tumefaciens, P. campestris, P. phaseoli, P. syringae, P. malvacearum, P. flaccumfaciens, P. medicagensis and *P. phaseoli* var. *fuscus*; animal pathogens such as *Salmonella suipestifer, S. pullorum, S. enteritidis, Escherichia coli* and *Pseudomonas aeruginosa*; and human pathogens such as *Eberthella typhosa, Shigella dysenteriae* "Flexner," *S. dysenteriae* "Strong," *Salmonella schottmuelleri, S. paratyphi,* and *Staphylococcus citreus*.

3. In the majority of cases the colonies of associated bacteria were completely consumed by the myxamoebae which constitute the vegetative stage of this and related slime molds; in a few cases the bacterial colonies were only partially consumed.

4. Using other media than hay-infusion agar, *D. discoideum* was grown upon three additional human pathogens, namely: *Staphylococcus aureus, Bacillus anthracis,* and *Corynebacterium diphtheriae*.

5. Other species of the Dictyosteliaceae such as *Dictyostelium mucoroides, D. purpureum* and *Polysphondylium violaceum,* which are widely distributed in nature, are likewise capable of feeding upon a variety of pathogenic bacteria.

6. Because of the ease with which they can be identified and maintained in culture, species of the Dictyosteliaceae afford excellent material for experimental studies relating to the nutrition and feeding habits of amoeboid cells.

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PLATE 1

FIGS. 1 to 6. Successive stages in the consumption of colonies of *Escherichia coli* by *Dictyostelium discoideum*. × 6.

FIG. 1, bacterial colony prior to inoculation with *Dictyostelium*.

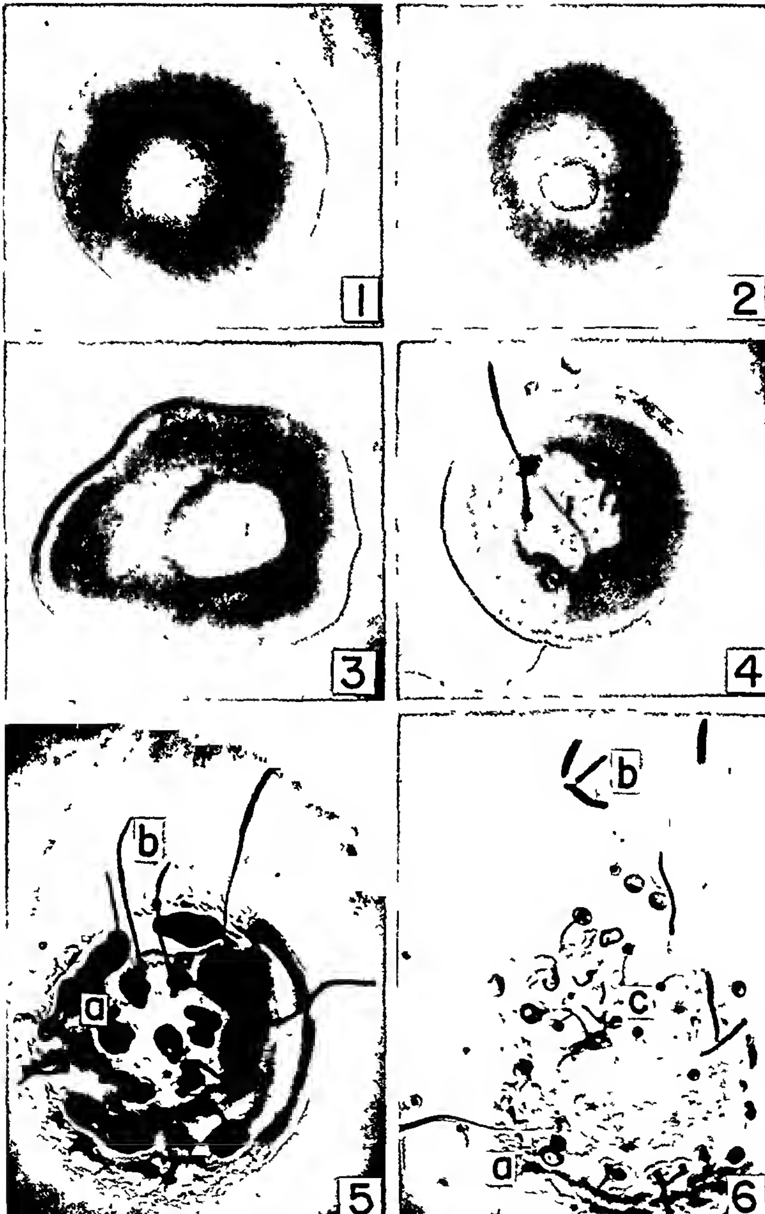
FIG. 2, 24-hour culture of *D. discoideum* showing central area of bacterial colony consumed by the slime mold.

FIG. 3, 32-hour culture showing increased clearance of bacterial colony.

FIG. 4, 48-hour culture; early fruiting stages of *Dictyostelium* evident.

FIG. 5, 54-hour culture showing increased growth and further development of *D. discoideum*. *a*, forming pseudoplasmodia; *b*, migrating pseudo-plasmodia (early stage).

FIG. 6, 72-hour culture; bacterial colony almost wholly consumed; fruiting structures of *Dictyostelium* abundant: *a*, forming pseudoplasmodia, *b*, migrating pseudoplasmodia (later stage), *c*, mature sorocarps, sori or spore heads not in focus.



(Kenneth B Raper and Nathan R Smith Growth of *Dictyostelium discoideum*)

PLATE 2

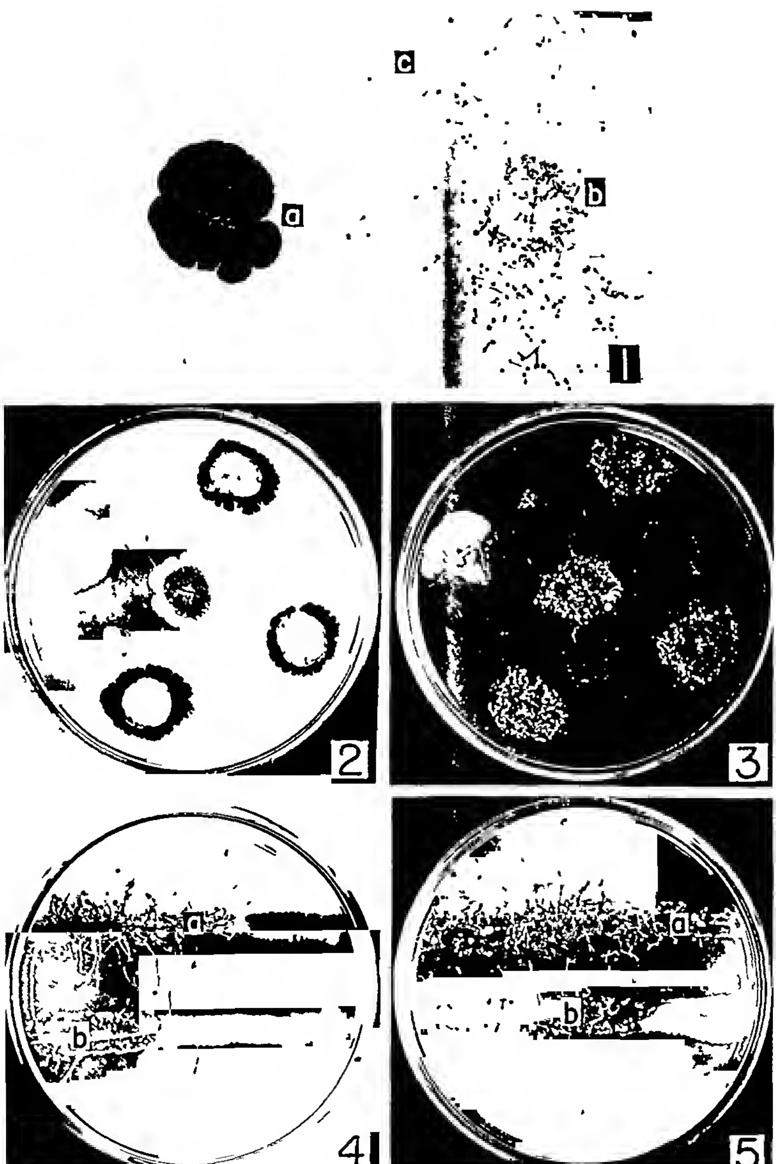
Fig 1 *Dictyostelium discoideum* growing in association with *Escherichia coli* upon buffered peptone agar *a*, uninoculated bacterial colony *b*, colony similar to *a* 10 days after inoculation with *Dictyostelium* *c*, isolated sorocarps bearing bacteria-free spores × $1\frac{1}{4}$

Fig 2 Three-day culture of *D discoideum* in association with *Eberthella typhosa* upon carrot-peptone agar colony at left uninoculated with *Dictyostelium* × $\frac{1}{2}$

Fig 3 Culture shown in figure 2 ten days after inoculation with *D discoideum* Note the completeness with which inoculated colonies have been consumed and the present invasion of the control colony by the slime mold × $\frac{1}{2}$

Fig 4 Four-day culture of *D mucoroides*, *a*, and *D discoideum*, *b*, in association with *E typhosa* upon carrot-peptone agar Slime mold spores inoculated at left end of bacterial streaks × $\frac{1}{2}$

Fig 5 Five-day culture of *D mucoroides* *a*, and *D purpureum*, *b* in association with *E typhosa* × $\frac{1}{2}$



(Kenneth B Raper and Nathan R Smith Growth of *Dictyostelium discoideum*)

A STUDY OF STREPTOCOCCI PRODUCING POSITIVE HOTIS REACTIONS[1]

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The Hotis test, described by Hotis and Miller (1936) as a simple method for detecting mastitis streptococci in milk, is attractive to veterinarians and milk control officials because the technic is relatively simple and positive tests are easily read. To 0.5 ml. of a 0.5 per cent sterile, aqueous solution of the indicator dye, brom-cresol-purple, in a sterile test tube is added 9.5 ml. of the milk, and this is incubated for 24 hours at 37.5°C. According to the originators of the test, "If streptococci are present, the color changes from purple to a yellow shade during incubation as a result of the production of acid from lactose by these organisms. In addition to this change, if *S. agalactiae* is present, small flakes or balls of growth, from 0.5 to 4 mm. in diameter, usually form on the side of the tube."

Hotis had earlier noted a tendency of *Streptococcus agalactiae* to grow in clumps, and Devereux (1935) wrote, "In making brom thymol blue keeping-quality determinations of milk on quarter, cow samples, and producer samples, it was noted that a flocculent growth frequently occurred. This was typical of the type of growth often produced by streptococci. Microscopic examinations verified these findings in that either short or long chain streptococci were present in large numbers when this type of growth was noted. However, there was no apparent difference in the growth produced by *Streptococcus lactis* and the mastitis organisms. . . It was also found that only about half of the

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samples that were culturally positive for mastitis streptococci displayed this flocculent growth. . ."

COMPARISON OF THE HOTIS TEST WITH OTHER TESTS FOR MASTITIS

Hotis and Miller found the Hotis test to be in perfect agreement with the blood-agar method in 715 of a series of 753 samples, and they obtained mastitis streptococci on blood-agar plates from every one of the 560 samples which gave flakes and typical color changes. This led them to conclude that, "A characteristic change in the color of the sample after incubation together with the occurrence of flakes or balls of growth indicates the presence of *S. agalactiae*."

TABLE 1

Correlation of pH, catalase, chloride, and Hotis tests in quarter samples from the Wash. Agric. Expt. Sta. herd

NUMBER OF QUARTERS WITH		NUMBER OF SAME QUARTERS WITH			
		pH over 6.8	Catalase over 3 ml. gas	Chlorides over 0.1595 per cent	Hotis + reaction
pH over 6.8	49		21	27	1
Catalase over 3 ml. gas	27	20		20	1
Chlorides over 0.1595 per cent	41	27	21		1
Hotis + reaction	4	1	1	1	

Bryan and Devereux (1937) compared the results obtained by direct microscopic examination, blood-agar plates, and the Hotis test. The blood agar detected 89 per cent of the composite samples positive to the microscopic test as compared with 92.5 per cent when quarter samples were tested. Considering the results of the microscopic test as accurate in detecting the presence of mastitis streptococci, they found the Hotis test, after 24 hours incubation, detected from 52.8 to 64.3 per cent of the positive composite samples; after 48 hours, from 62.2 to 71.4 per cent. The correlation was somewhat better with quarter samples, being 64.3 per cent after 24 hours and 78.6 per cent after 48 hours incubation. However, the longer period of incubation produced so many suspicious reactions that the results were difficult to interpret.

When studies were made of quarter samples from the dairy herd of the State College of Washington at Pullman, the results of the Hotis test did not correlate with those obtained by determining the pH either electrometrically or colorimetrically, the catalase test basing a positive reaction on the formation of three or more cubic centimeters of gas, or the chloride test using 0.1595 per cent of chlorides as the maximum in normal milk. All but four Hotis tests were negative, although clinical mastitis occurs in this herd with moderate frequency.

Quarter samples from the herd of the Western Washington Experiment Station at Puyallup revealed a larger percentage of Hotis positives, although the incidence of clinical mastitis in this herd is low.

PURE CULTURE STUDIES OF HOTIS-POSITIVE AND HOTIS-NEGATIVE QUARTERS

In order to ascertain whether or not the positive Hotis tests obtained were due to the presence of *Streptococcus agalactiae* and whether this organism was present in quarters yielding abnormal milk but which gave negative Hotis reactions, studies were made of pure cultures from both normal and abnormal quarters yielding positive Hotis tests and from abnormal quarters yielding negative Hotis tests. The cultures were obtained by streaking fresh quarter samples, incubated quarter samples, or the flakes from typically positive Hotis tests on blood agar plates and picking typical colonies into litmus milk or glucose veal-infusion broth. To ascertain whether or not the pure cultures so obtained would produce typically positive Hotis reactions when grown in a suitable environment, they were cultured in aseptically-drawn brom-cresol-purple milk. This milk, obtained from quarters consistently negative to the Hotis reaction, consistently gave typical Hotis reactions when inoculated with a known strain of *Streptococcus agalactiae.*

The use of unsterilized milk, however, as a culture medium for these studies was recognized as unsound since aseptically-drawn milk rarely is sterile. The necessity of avoiding the use of unsterilized milk was further emphasized by Hucker (1937) who

reported that, "A study of twenty-four udders aseptically removed and cultured from cows known to be free of mastitis and to have passed through one or more lactation periods shows that all contained mastitis streptococci."

When autoclaved milk was substituted for the raw milk, only an occasional tube had the typical flakes on the side, although the organisms grew readily and produced sufficient acid to alter the color of the indicator. Varying the concentration of the inoculum, changing the dye concentration, adjusting the pH to 6.8, 7.0, or 7.2 with disodium acid phosphate, raising the chloride content to a total chloride concentration of 0.16 per cent, or adding calcium to compensate for possible precipitation during sterilization failed to enable the milk to support a positive Hotis reaction. Symbiotic relationships between various milk organisms were improbable because, when typical flakes from positive reactions were transferred to autoclaved brom-cresol-purple milk, they failed to give positive Hotis reactions.

The altered chemical composition of mastitic milk is due principally to infiltration of blood elements through the injured mammary tissue. From 10 to 15 per cent of blood serum from either equines or bovines therefore was added to the autoclaved brom-cresol-purple milk to raise the pH to approximately 7.0. Inoculation with a known Hotis-positive strain of streptococci and incubation for 24 hours produced typical flakes and color changes in every case.

It was evident, therefore, that heating inactivated or destroyed some factor in fresh milk which was responsible for the tendency of certain streptococci to grow in flakes on the side of a test tube and that blood serum either reactivated the milk or supplied that which had been destroyed.

THE RÔLE OF AGGLUTININS IN THE HOTIS REACTION

Normal milk which did not give a Hotis reaction but which supported the Hotis reaction when inoculated with a certain strain of streptococci, was divided into two parts. To both parts was added 0.025 per cent brom-cresol-purple and to one was added 20 per cent equine blood serum known to be capable of

activating the Hotis reaction with the strain of streptococci used in this experiment. Portions of each were subjected to various degrees of heat for different periods of time. After cooling, they were inoculated with a strain of streptococci known to produce large flakes in the Hotis reaction and were incubated.

Table 2, the composite of three trials, shows that exposure to 80°C. for five minutes destroys, and exposure to 70°C. for approximately 30 minutes weakens the ability of milk or milk-serum mixtures to support the Hotis reaction. This is within the range of time and temperature which inactivates agglutinins. According to Jordan, "Agglutinin is weakened by heating to from 60 to 70°C. and is destroyed at 75°C. With serum heated to 78°C., no agglutination appears." It also will be noted that milk-serum mixtures were slightly more thermo-tolerant than milk alone, probably because the heat destruction of agglutinins is a gradual process and the serum supplied a greater initial amount.

The more intense reaction in milk and milk-serum mixtures heated to 60° than to 50°C. probably is due to the destruction of the complement and the inactivation of the bactericidal properties of the blood serum and milk at 60°C. The streptococci studied uniformly grew more rapidly in heated than in fresh milk and grew least rapidly in fresh milk to which blood serum had been added.

Early bacteriologists recognized the tendency of streptococci, pneumococci, and certain other organisms to grow in clumps when the medium contained agglutinins specific for them. The reaction was first reported by Charrin and Roger (1889) with *Pseudomonas pyocyaneus*, and Pfaundler (1898) made similar observations with *Escherichia coli* and *Proteus vulgaris*. This phenomenon has been used for the identification of the pneumococci which are made to grow in long threads, often arranged in tangled masses. The process is essentially one of agglutination during growth. In describing this phenomenon, Arkwright (1931) wrote, "An appearance seen in young cultures grown in specific immune serum diluted with broth, which is known as the thread reaction, is almost certainly due to the same causes as

TABLE 2

Effect of heat on the Hotis activating ability of milk and serum

	NORMAL MILK						NORMAL MILK PLUS 20 PER CENT SERUM					
	Time in minutes											
	5	10	15	20	30	60	5	10	15	20	30	60
Not heated	++						++					
50°C	++	++	++	++	++	+++	++	++	++	++	++	++
60°C	++++	++++	++++	++++	++++	+++	+++	++++	++++	++++	++++	++++
70°C	++++	+++	+++	+++	++	+	++++	++++	++++	+++	++	++
80°C	−	−	−	−	−	−	−	−	−	−	−	−

−, no flakes on the side of the tube.
+, one large flake or from one to five small flakes on the side of the tube.
++, two to five large flakes or five to ten small flakes on the side of the tube.
+++, five to ten large flakes or numerous small flakes on the side of the tube.
++++, very numerous large or small flakes on the side of the tube.

produce somatic or capsular agglutination, acting during the growth and division of the bacteria in the presence of agglutinins." The same alteration of the surface which causes bacteria to clump would tend to make them migrate toward any interface, in the Hotis test the wall of the tube, and adhere.

Further evidence that agglutinins are responsible for the characteristic flake formation on the side of the Hotis tube is seen in the fact that the titre of any serum for activating the Hotis reaction could be markedly reduced against any one serological group by agglutinin absorption methods.

Spontaneous agglutination of many strains of organisms has long been recognized. Some strains of *Streptococcus agalactiae* used in this work occasionally produced flakes on the side of the tube of autoclaved brom-cresol-purple milk when incubated without serum. Recently, a beta-hemolytic streptococcus isolated from a case of scarlet fever grew in flakes, typical of those of the Hotis test, on the side of a tube of glucose broth. The work done in this experiment, however, shows that only rarely are the forces responsible for spontaneous agglutination sufficient to give rise to the formation of flakes or balls on the side of a tube of autoclaved milk.

DISTRIBUTION OF HOTIS-ACTIVATING AGGLUTININS

Agglutinins capable of activating the Hotis reaction appear to be widespread. Blood sera from over 90 per cent of the Hotis-negative cows, from all of the Hotis-positive cows studied, from all of several horses which served as sources of blood, from all of six cavies, two humans and one chicken were capable of supporting typical flake formation when added to autoclaved brom-cresol-purple milk and inoculated with *Streptococcus agalactiae* or other streptococci capable of causing typical reactions.

In the herd of the State College of Washington, all quarter samples from 18 Hotis-negative cows supported the Hotis reaction when inoculated with a pure culture of streptococci obtained from a positive Hotis reaction. None of the uninoculated controls gave a positive reaction nor did the culture when inoculated into autoclaved brom-cresol-purple milk.

However, aseptically drawn unheated milk from an experimental herd free from any known carriers of udder streptococci, failed to support the Hotis reaction when inoculated with *Streptococcus agalactiae*. When human blood serum was added at the time of inoculation, typical reactions developed after incubation. Presumably, the milk from the animals in this experimental herd was free from agglutinins for this group of streptococci.[2]

DISTRIBUTION OF HOTIS-POSITIVE STREPTOCOCCI

The widespread distribution of Hotis-activating agglutinins suggested an equally wide distribution of Hotis-positive streptococci. Throat swabs were taken from 155 students and inoculated into sterilized brom-cresol-purple milk plus ten per cent of either equine or human blood serum and incubated 18 to 24 hours. Of these, 140 gave typical positive Hotis reactions, 12 were negative, and three questionable. All contained streptococci and when streaked on blood agar and incubated, yielded weakly hemolytic colonies which could not be distinguished from those of *Streptococcus agalactiae*. These data indicate that streptococci from most human throats will produce the Hotis reaction when grown in the presence of human or equine blood serum.

Further trials were made to ascertain whether streptococci from human throats would produce typical Hotis-positive reactions when grown in unheated milk. Twelve of the throat swabs which yielded positive results in the previous experiment were used to inoculate normal, Hotis-negative milk from a single quarter. In the absence of added blood serum, four of these yielded positive Hotis reactions. This would indicate that this apparently normal quarter produced milk which contained agglutinins for streptococci from at least four of twelve human throats.

In another trial, milk samples from seven apparently normal Hotis-negative quarters were inoculated with throat swabs from one person and five of these after incubation gave positive Hotis reactions.

[2] The authors wish to express their appreciation to Dr. O. W. Schalm of the University of California for his cooperation in this portion of the experiment.

Fifteen nasal swabs from Hotis-negative cows yielded two Hotis-positives when inoculated into autoclaved milk plus serum. Microscopically and on blood agar these organisms had the appearance of *Streptococcus agalactiae* and they hydrolyzed sodium hippurate and failed to utilize esculin.

Vaginal swabs from 11 Hotis-negative cows and tears from four Hotis-negative cows did not give positive Hotis reactions when inoculated into autoclaved milk plus serum.

CULTURAL CHARACTERISTICS OF HOTIS-POSITIVE STREPTOCOCCI

Using a capillary pipette, typical flakes were fished from the side of Hotis-positive tubes into 2 ml. of glucose broth. After four hours incubation, the cultures were streaked on five per cent bovine blood agar and incubated over night at 37.5°C. Typical colonies were transferred to glucose broth and these incubated for 24 hours; then loop transfers were made into various media. The diversity of action of these streptococci on carbohydrates is shown in table 3.

The bovine strains most frequently isolated utilized sodium hippurate but not esculin. The strains from human sources, in general, utilized neither. In both types, however, there were variations, including human strains, that had the same reactions as *Streptococcus agalactiae*; i.e., utilization of sodium hippurate but not esculin.

Thirty-seven cultures of streptococci isolated from cows and 61 isolated from human beings were identified serologically using the Lancefield technique as modified by Brown and in addition were grown in esculin and sodium hippurate broths, and in sterilized brom-cresol-purple milk plus ten per cent serum. Most human cultures were grown in the same serum which was used for the original Hotis test, but many of these did not produce typical flakes in pure culture until grown in a different serum-milk mixture. Not all pure cultures of bovine origin produced positive reactions in any one serum.

From table 4 it will be seen that most of the bovine strains isolated from flakes in positive Hotis tubes belong to Lancefield's serological group "B," and that all but five of these produced a

TABLE 3

Carbohydrate reactions of Hotis-positive streptococci

	SODIUM HIPPURATE	ESCULIN	SALICIN	MANNITOL	INULIN	ARABINOSE	RAFFINOSE
Strains from bovine sources	+	−	−	−	−	−	−
	+	+	+	+	+	+	+
	+	+	+	+	+	+	−
	+	+	+	−	+	+	+
	+	+	+	−	+	+	−
	+	+	+	+	+	−	−
Strains from human sources	+	−	−	−	−	−	−
	−	+	+	+	+	+	+
	−	−	+	−	+	−	−
	−	−	+	−	+	−	+
	−	−	−	−	+	−	+
	−	+	+	−	+	−	+
	−	−	+	+	+	+	+
	−	−	−	−	−	−	+

+, hydrolysis; −, no hydrolysis.

TABLE 4

Serological grouping of streptococci

BOVINE STRAINS					HUMAN STRAINS				
Number of strains	Group	P. C. Hotis	Sodium hippur.	Esculin	Number of strains	Group	P. C. Hotis	Sodium hippur.	Esculin
20	B	+	H	−	31	?	−	−	−
5	B	+	−	−	21	?	+	−	−
2	B	+	H	ac	1	?	−	−	ac
1	B	+	−	ac	5	B	−	H	−
2	B	−	H	ac	2	B	+	H	−
2	B	−	−	ac	1	A	+	H	−
1	B	−	H	−					
1	C	+	H	ac					
2	C	−	H	ac					
1	?	−	−	−					

Group = Lancefield's serological group; ? = not in Lancefield's serological groups "A," "B," or "C"; + = positive Hotis reaction in pure culture; H = hydrolysis; ac = acid.

positive Hotis reaction in pure culture when grown in the modified medium.

Of the human strains isolated from flakes in positive Hotis reactions from throat swabs in the modified medium, seven belonged to Lancefield's group "B," one to group "A," and fifty-three to neither "A," "B," or "C"; twenty-one of these belonging to some other than the first three groups, yielded positive Hotis reactions in pure culture when grown in the modified medium using equine serum.

As noted above, some of the cultures would not form flakes when grown in the same serum used in the original test, but would in another serum. Furthermore, any one culture might not form typical flakes in every one of a series of tubes using one serum, although clumps would appear in the bottom of the tube. This would suggest the presence of several groups of agglutinins and also that the migration of streptococci to the sides of the tube when grown in the presence of agglutinins specific for them is somewhat a matter of chance. Some strains, especially those which showed some tendency towards self-agglutination, produced typical flakes with more frequency than did certain other strains. The presence of several groups of agglutinins and perhaps the matter of chance in the migration to the sides of the tube is further suggested by the results obtained in the previously-mentioned series of throat swabs in normal milk without added serum.

DISCUSSION

The Hotis test appears not to be specific for *Streptococcus agalactiae*. Even when attempts were made to confirm the identity of the organisms by streaking Hotis-positive milk on blood agar and selecting only the weakly hemolytic colonies for further study, many diverse streptococci were included. It is evident that the combined use of the Hotis test and blood agar plates is not sufficient for the differential diagnosis of *Streptococcus agalactiae*.

The presence of weakly hemolytic, Hotis-positive streptococci in most human throats and in the nasal discharges of some Hotis-negative cows throws serious doubt on the assumption that the elimination from a milking herd of every cow that is

shedding such streptococci in the milk will result in the herd remaining free from streptococcic infection. Apparently, streptococci of this general class are much more ubiquitous than formerly supposed.

In herds such as were available to the originators of the Hotis test, in which the organism most frequently associated with mastitis is *Streptococcus agalactiae*, the Hotis test may continue to have some value. In other herds, the results may be confusing or misleading. The identification of an organism by means of an agglutination reaction, in which both the organism and the agglutinating serum are obtained from the same clinical case, must be recognized as scientifically questionable.

SUMMARY AND CONCLUSIONS

The Hotis test has been described as being specific for *Streptococcus agalactiae*, but in this work, using both unheated milk and sterilized Hotis-negative milk plus blood serum, it has been found that many organisms, of the genus *Streptococcus* at least, are able to produce the reaction.

The Hotis reaction appears to be an agglutination reaction and the alteration of the surface which causes the organisms to clump would cause them to tend to migrate to the surface of the test tube and adhere.

It appears that the yellow flakes or balls which form on the side of a tube of brom-cresol-purple milk after incubation, and which have been considered diagnostic of *Streptococcus agalactiae* in the Hotis test, may be produced by any organism which:

1. Stimulates the production of agglutinins.
2. Grows in the presence of 0.025 per cent brom-cresol-purple in a milk medium when incubated in a test tube under aerobic conditions at 37.5°C.
3. Forms clumps on the side of the tube when grown in the presence of its agglutinins.
4. Produces sufficient acid from lactose to increase the hydrogen ion concentration to about pH 5.4.

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COLONY AND ANTIGENIC VARIATION IN KLEBSIELLA PNEUMONIAE TYPES A, B AND C

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INTRODUCTION

It is commonly recognized that *Klebsiella pneumoniae is* closely related to the *Escherichia-Citrobacter-Aerobacter* group of gram-negative bacteria, the main differential factor being the capsule which is formed by *Klebsiella pneumoniae*. However, *Klebsiella pneumoniae* is usually placed in a separate category because its cultural reactions are not typically those of either the *Escherichia* group or the *Aerobacter* group, and also because it is often associated with pathological conditions in man and animals. The precise relationship of this organism to the *Escherichia*, *Citrobacter*, and *Aerobacter* groups remains to be established.

The confusion concerning this relationship is emphasized by the fact that Edwards (1929) showed that many cultures ascribed to the genus *Aerogenes* are serologically identical with certain strains of *Klebsiella pneumoniae*. This relationship was studied further by Julianelle (1937), who found that some strains of encapsulated *Aerogenes* cross-agglutinate with type B of Friedlander's bacillus, and with type II pneumococcus in serological tests. However, absorption tests revealed differences between the capsular material of the type B Friedlander's bacillus and *Aerobacter aerogenes*.

Obviously, the presence or absence of a capsule does not constitute a reliable criterion for the separation of *Klebsiella pneumoniae* from the members of the Colon-Intermediate-Aerogenes group.

All members of the coli form group, may, under certain conditions assume a mucoid form. Revis (1910) caused a colon

organism to develop a mucoid form of growth by cultivating it in sterilized soil. Gratia (1922) reported the development of mucoid colonies of colon bacilli when non-mucoid types were subjected to the action of bacteriophage. Smith (1925) found a mucoid colon organism connected with calf scours, and Parr (1933) has repeatedly isolated mucoid colon organisms from normal feces.

Furthermore, experience shows that Friedlander's bacilli, when decapsulated, exhibit a smooth, translucent colony form characteristic of the colon bacillus. This is a form which Julianelle in his studies on dissociation of *Klebsiella pneumoniae* did not describe.

In an investigation of the colony variants of *Klebsiella pneumoniae*, Julianelle (1928) reported direct transition from the smooth, mucoid colony form to the rough colony form when cultures of the mucoid bacilli were grown in homologous immune serum. The transformation of *Klebsiella pneumoniae* from the mucoid colony type to the rough colony type was also reported by Hadley (1925) and O'Neal (1933).

Dawson (1934) pointed out that there exist three main colonial forms within the species *Diplococcus pneumoniae*. These are mucoid, smooth and rough. The mucoid colony consists of encapsulated, virulent organisms. The smooth colony consists of organisms which have lost their virulence and their capsules but which form stable suspensions in broth. The rough colony consists of organisms which are non-encapsulated, avirulent, and which form a sediment in broth.

This paper presents evidence that an analogous situation more accurately describes the variants of Friedlander's bacillus, and that capsules and colonial form cannot be relied upon to separate Friedlander's bacilli from other coliform bacteria.

DISSOCIATION OF KLEBSIELLA PNEUMONIAE[1]

The three serological types, A, B, and C of *Klebsiella pneumoniae*, in mucoid form were seeded into 0.5 per cent lithium

[1] The strains used in this work were obtained from Dr. John Hanks of George Washington University and from Dr. L. A. Julianelle of Washington University. They were Type A Strain Sc., Type B Strain E., and Type C Strain F10

chloride peptone water and incubated at 37°C. for one week. At this time plates of meat infusion agar were streaked and the resulting colonies examined for any variation from the normal. When colonies differing from the original mucoid type were observed some were picked, reseeded into lithium chloride peptone water and again incubated for one week. At each picking several variant colonies were selected for morphological and biochemical study. This process was continued until the variants to be described later were obtained.

The dissociative process took place at a different rate for each of the types A, B, and C, but the first step in each case consisted in a change from the mucoid colony form to a translucent colonial variant which remained stable in subculture on meat infusion agar. The second step was the gradual roughening of the translucent variants until typical rough variants were obtained. Type A gave rise to translucent variants after three weekly transfers. Representative translucent colonies were again transferred to lithium chloride peptone water and produced stable rough colonies after six weekly transfers. Type B produced translucent variants after three weekly transfers. Colonies of this translucent form produced rough colonies after four weekly transfers in lithium chloride peptone water. Type C gave rise to translucent variants after two weeks. These variants gave rise to rough colonies after three more weekly transfers in lithium-chloride peptone water. The mucoid, translucent, and rough colony forms of each strain have been kept on meat infusion agar for the past four years without further detectable variation.

When types A and C gave rise to translucent variants, there also appeared very small dew drop colonies. When they were replated these dew drop colonies gave rise to translucent forms or to a mixture of translucent and dew drop colony types. The type C translucent colonies were purified so that they no longer produced dew drop colonies but the translucent variant from type A still retains this tendency after four years.

The translucent variant derived from type A grows poorly on agar and in broth. In twenty-four-hour agar cultures, the colony size varies from very small dew drop colonies to larger forms. Sometimes, growth fails to occur unless a large inoculum

is used or else it is delayed from 36 to 72 hours. The rough variant of type B (Br) obtained from the translucent variant of type B (Bt) is also difficult to grow. Large inocula are necessary to initiate growth if the culture has been stored for several weeks in the ice box. Growth occurs more readily if blood agar is used. However, if the culture is transferred for several generations at 37°C. on meat infusion agar it will grow fairly profusely in 24 hours. The colonies produced vary in size from pin head types to forms 5 or 6 mm. in diameter.

The colonial appearance of the translucent variants resembled very strikingly the colonial form of *Escherichia coli* and other non-capsulated members of the coli-form group.

DESCRIPTION OF THE COLONY VARIANTS ON INFUSION AGAR AFTER 24 HOURS INCUBATION AT 37°C.

For ease in referring to the original colonies and variants, an abbreviated system of notation was used: As, type A, smooth and mucoid; At, translucent variant derived from type As; Ar, rough variant representing further dissociation of At. The same system was applied to types B, and C, i.e., Bs, Bt, Br; and Cs, Ct, and Cr.

Description of colonies: 1. As: Mucoid, opaque, white, about 3 mm. in diameter; round, entire edge, surface smooth, glistening and convex; viscous consistency.

2. At: Non-mucoid, translucent, greyish white; smaller than As; round, entire edge; surface smooth, glistening, and slightly convex; butyrous. There is considerable variation in the size of the colonies.

3. Ar: Rough, translucent, greyish white, about 2 mm. in diameter; round with a crenated edge, surface slightly dull, and flat with a thin, narrow periphery; butyrous.

4. Bs: Similar to As except the colonies appear more of a pasty white.

5. Bt: Similar in appearance to the larger colonies of At. The colonies of Bt are of uniform size, 2 to 3 mm. in diameter.

6. Br: Rough, opaque, dirty-white frosted glass appearance; size varies from small to quite large; round with a crenated edge;

surface dull and flat; not viscous, but tough and difficult to emulsify. On ageing, the colonies appear to enlarge by spreading a thin growth outward from the raised center.

7. Cs: Similar to Bs.

8. Ct: At first gave rise to the dew drop colony forms, but later became stabilized and now resembles Bt.

9. Cr: Rough, opaque, dirty-white ground-glass appearance; about 2 to 3 mm. in diameter; the colonies are often of an irregular oval shape; edge crenated; surface dull and flat; not viscous, but difficult to emulsify.

MORPHOLOGY AND STAINING CHARACTERISTICS OF THE ORIGINAL TYPES AND THEIR VARIANTS

The organisms in the 24-hour mucoid colonies of all three types were plump, gram-negative rods, occurring singly or in pairs. Capsules were readily demonstrated when these organisms were emulsified in serum and stained with Wright's stain.

All of the translucent variants were very similar in morphology to each other. They were short, gram-negative, non-encapsulated rods.

The rough variants did not possess capsules and differed from the mucoid and translucent varieties in appearing as long rods or filaments. Certain individual differences were noted. The rough variant of type A was a gram-negative organism occurring as a mixture of rather long rods, together with many shorter forms resembling the organisms from the translucent colonies. Br was very long and filamentous and showed some evidence of branching. The cytoplasm was granular and gram-negative with some sections of the rods being gram-positive. Cr was a long gram-negative rod, longer than Ar, but not as filamentous as Br.

CULTURAL AND BIOCHEMICAL REACTIONS

1. Growth in broth

Within 24 hours all transplants of the three mucoid cultures produced a diffuse turbidity in broth with a ring at the surface and a viscid sediment. The translucent variants grew with a uniform turbidity and produced a slight non-viscid sediment

in 24 hours. A ring at the surface appeared only after cultivation for a period of two to five days. The rough variant of type A (Ar) grew with a diffuse turbidity and a heavy pellicle which settled to the bottom on slight agitation to form a heavy granular sediment. The Br variant grew slowly in broth, producing a very faint turbidity and a fluffy sediment at the bottom of the tube. Upon prolonged incubation, the Br culture grew entirely at the bottom of the tube, leaving a clear supernatant. The Cr variant produced a faint turbidity, a slight surface film, and grew chiefly as a sediment at the bottom of the tube.

TABLE 1

ORGANISMS	2 DAYS*		4 DAYS VOGES-PROSKAUER	21 DAYS		7 DAYS REDUC. OF NITRATES	21 DAYS	
	Indol	Methyl red		Citrate	Gelatin		Glucose†	Lactose
As.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
At.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A-alk.
Ar.......	Pos.	Pos.	Neg.	Pos.	Neg.	Pos.	AG	A
Bs.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
Bt.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
Br.......	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.
Cs.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	AG	AG
Ct......	Neg.	Neg.	Neg.	Pos.	Neg.	Pos.	AG	AG
Cr.......	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.

* This refers to the incubation period at 37°C.

† The reactions of the various strains in sucrose and mannitol are the same as those recorded in glucose.

2. *Biochemical reactions*

During the course of this work, the different strains showed some variation in their Methyl Red and Voges-Proskauer reactions. In 1925 Small and Julianelle found that most of their strains of *Klebsiella pneumoniae* were Methyl-Red positive and Voges-Proskauer negative and that these reactions were of no value in the classification of this group of organisms. The original strains and the variants used in this study were Methyl-Red positive and Voges-Proskauer negative, with the exception of Br and Cr which fermented no sugars and, therefore, were negative in both tests.

On Koser's citrate medium, all strains, except Br and Cr, grew and utilized the citrate.

With the exception of the rough variant of type A all strains failed to produce indol.

All the cultures and variants, except Cr, reduced nitrates to nitrites. The variant Cr also was the only strain or variant which liquefied gelatin.

The reactions on several carbohydrate substrates (see table 1) were uniformly constant during the year or more during which the original types and the variants were under frequent observation. This has been an aid in checking the purity of the different strains during this investigation. The translucent variants of all three mucoid types had the same biochemical reactions as the parent strains.

The rough strains showed marked cultural differences from the mucoid and translucent strains. The Ar variant differed from the parent strain in producing gas in glucose, sucrose and mannitol and in producing indol. The Br variant was inactive on all test substances except nitrates. The Cr variant lost its ability to ferment sugars and to reduce nitrates, but gained the power of liquefying gelatin.

AGGLUTINATION REACTIONS

Suspensions for the agglutination test and for animal inoculation were prepared by growing the organisms on meat infusion agar, washing off the growth with saline solution, and heating the suspensions at 60°C. for one hour. Sterility tests were then made and the suspensions stored in the ice box. Agglutination tests were incubated for 2 hours at 52°C.

Julianelle (1926) (1937) reported that rough strains derived from different types of Friedlander's bacilli cross agglutinate with one another, producing a granular type of agglutination. This granular type of agglutination is considered by Julianelle to be characteristic of rough organisms which lack the specific polysaccharide, while specific agglutination, which is due to the capsular material, is like a precipitin reaction and produces a cake in the bottom of the tube. Bamforth (1928) in an investigation of the

Capsulatus-mucosus group, reported that he obtained specific agglutination of a granular type with some of the strains he investigated. Consequently, he did not believe that specific agglutination was necessarily of the "compact-disc" type.

In the present work, three kinds of agglutination have been observed. First, the compact-disc type which is seen when encapsulated bacilli are agglutinated by their specific antisera. Second, granular agglutination which was observed with all the translucent variants and their specific antisera and third, a "fluffy" type of agglutination observed when the rough variants Br and Cr were acted on by their specific antisera. In the latter

TABLE 2

Antisera

ORGANISMS	As	At	Ar	Bs	Bt	Br	Cs	Ct	Cr
As........	10*								
At........	80	640							
Ar........		160	640						
Bs........				20					
Bt........				160	1,280				
Br........						640			
Cs........							10		
Ct........							80	640	
Cr........									2,560

* This number represents the highest dilution of antisera giving an easily discernible reaction.

type of agglutination, the organisms settled rapidly to the bottom of the tube, and, when shaken resuspended without the formation of granules.

The agglutination reactions reveal (See table 2) that the dissociants do not cross react with variants from strains belonging to the other serological types.

The serological reactions of Ar are different from the reactions of the other rough variants. While the Br and Cr variants are specific and agglutinate only with the homologous anti-serum, Ar is agglutinated by antisera against At, although to a lower titer than by its own (homologous) antiserum.

DISCUSSION

The results obtained in this study indicate that it is possible to obtain from mucoid encapsulated *Klebsiella pneumoniae* smooth translucent colony variants which resemble the colonial form of *Escherichia coli*. It is from these translucent variants that one derives the rough colony type described by Julianelle (1928), which resemble rough variants of other members of the coli-form group. Furthermore, not only do the translucent colonies look like those of *Escherichia coli* but if acid and gas are produced in carbohydrate media, as in the case of Ct, it is impossible, because of the utilization of citrate, to distinguish the organism from some of the members of the *Citrobacter* group.

The similarity between the translucent variants of *Klebsiella pneumoniae* and organisms of the *Citrobacter* group is shown by their essentially identical colony morphology and biochemical reactions. Still another analogy can be found in the serological reactions of the translucent variants and the serological heterogeneity of the coli-form group.

Lovell (1937) described similar translucent variants produced by ageing cultures of encapsulated colon bacilli from "white scours" of calves and from one strain of *Aerobacter aerogenes*. Some of his translucent strains were serologically specific, while others reacted with each other in agglutination and precipitation tests. The grey (translucent) colonies described by Lovell were smooth and were composed largely of non-encapsulated organisms. It appears that the translucent variants of Lovell and the variants of *Klebsiella pneumoniae* described here correspond more closely with the smooth non-capsulated variant of the pneumococcus which Dawson (1934) described. Dawson's suggestion that the "R-1" colony variant described by Julianelle (1928) corresponds to the smooth, non-encapsulated colony form of the pneumococcus is not supported by the observations reported in this work.

Julianelle (1937) has reported that the rough variants of several serological types of *Klebsiella pneumoniae* cross-react. The results of this investigation do not confirm the occurrence of cross-

reactions among the rough variants of encapsulated *Klebsiella pneumoniae*. Perhaps, the use of two different means of inducing dissociation explains the different results, and, as shown by Julianelle, even rough variants derived from the same serological type of Friedlander's bacillus are not always serologically identical.

SUMMARY

1. By growing mucoid encapsulated strains of *Klebsiella pneumoniae* bacilli types A, B, and C in lithium chloride peptone water two distinct colony variants have been obtained from each type. These are designated "translucent" and "rough" forms.

2. The translucent colony variant is smooth and composed of non-encapsulated short rods resembling those of the parent strain.

3. The translucent variant has always been the first to appear in the dissociative process.

4. The rough variant was derived from the translucent type and produces a rough appearing colony which is composed of non-capsulated long rods or filaments.

5. The biochemical reactions of the original types and the derived variants have been recorded and their relations to the *Escherichia*, *Citrobacter*, and *Aerobacter* groups discussed.

6. The serological reactions of the original types and the variants derived from them have revealed a marked type specificity and in some cases an individual specificity within the type.

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PLATE I

Fig. 1. Colonies of Ar; 24 hours.
Fig. 2. Colonies of Br; 24 hours.

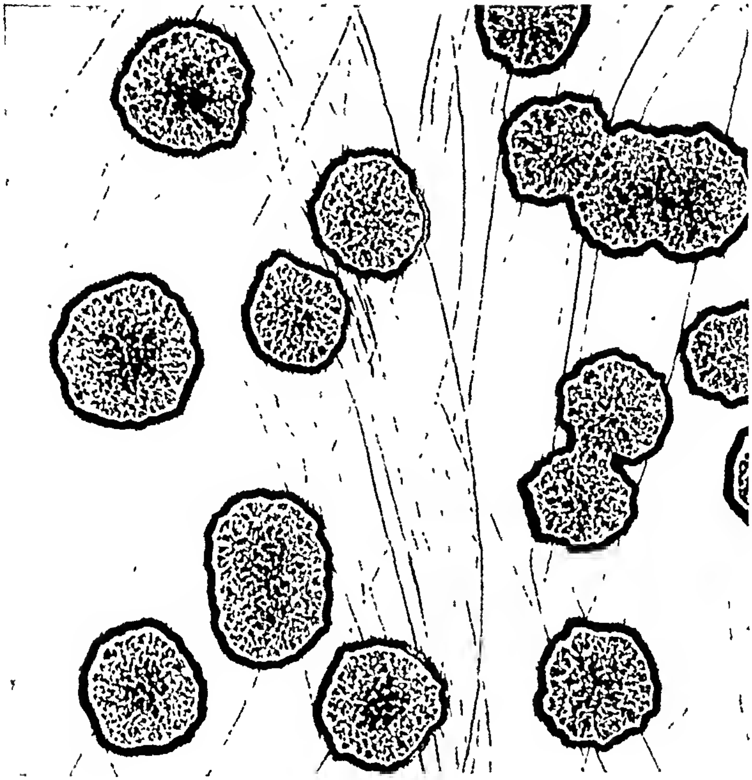

FIG. 1

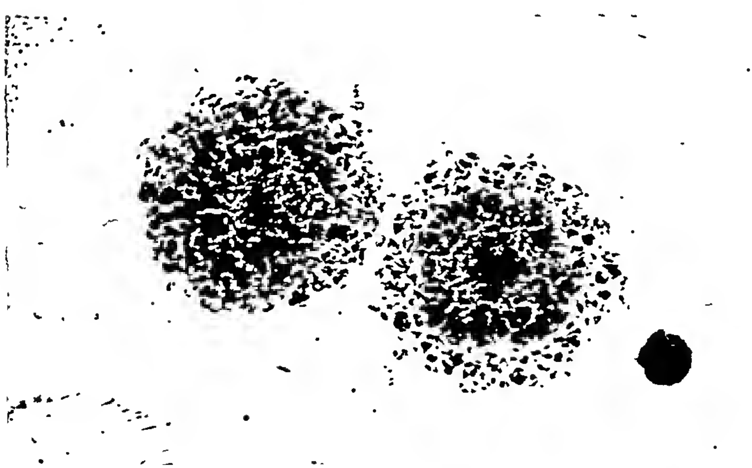

PLATE 1—*Continued*

FIG. 3. Colonies of Cr. 24 hours

FIG. 4. Gram stain of organisms in Br colony 24 hours

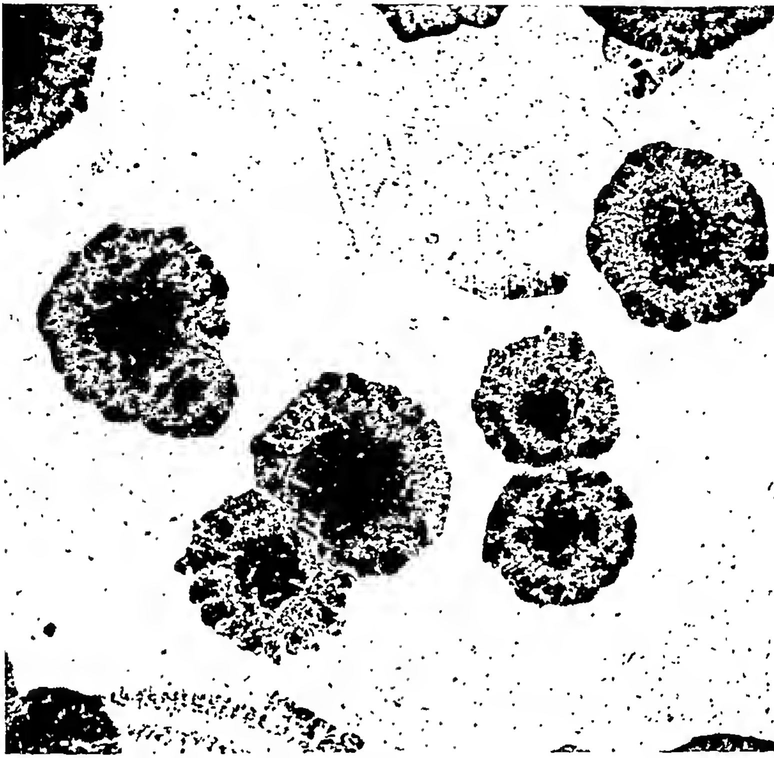

FIG. 3

PLATE 1—*Continued*

FIG. 5. Colonies of B translucent and B smooth and mucoid, 24 hours

FIG 5

(William A Randall Variation in Klebsiella Pneumoniae)

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MISSOURI VALLEY BRANCH

UNIVERSITY OF NEBRASKA, LINCOLN, NEBRASKA, MAY 6, 1939

STUDIES IN BOTULINUS TOXIN TYPE B. *N. P. Sherwood and Monty Belot,* University of Kansas, Lawrence, Kansas.

This investigation was carried out to throw light upon the following:

1. To determine the effect of botulinus toxin on animals which are able to synthesize their own vitamin "C" in comparison with those which do not possess this property.

2. To determine the effect of vitamin "C" upon botulinus toxin *in vitro.*

3. To determine whether repeated injections of vitamin C will have any effect upon botulinus toxin *in vivo.*

The results may be summarized as follows:

1. It was shown that guinea pigs were quite susceptible to the botulinus toxin employed both *per os* and parenterally. The M.L.D. parenterally was 0.005 ml.

2. White mice, which are supposed to manufacture their own vitamin "C", were found to be susceptible to botulinus toxin when injected but refractory when the toxin was given by mouth. Starvation of the animals previous to feeding toxins failed to render them susceptible.

3. One wild rat remained well for three days after eating 350 guinea pig M.L.D.'s of botulinus "B" toxin but succumbed when a like amount was again fed to it.

4. White rats seemed not to be injured by the injection of 100 guinea pig M.L.D.'s of botulinus "B" toxin.

5. When two M.L.D.'s of botulinus toxin were mixed with varying amounts of vitamin "C" and incubated for one-half hour, there seemed to result some reduction in toxin potency.

6. The resistance of six guinea pigs that received daily injections of 50 milligrams of vitamin "C" for four consecutive days was not increased for botulinus "B" toxin.

IN VITRO TESTS OF THE PATHOGENICITY OF 114 STRAINS OF STAPHYLOCOCCI OF MILK ORIGIN. *Pauline A. Keller and J. Ralph Wells,* Kansas State Teachers College, Pittsburg, Kansas.

This investigation of 114 strains of staphylococci isolated from the milk of individual quarters of cows' udders involved: (1) *in vitro* evidences of pathogenicity, (2) correlation of *in vitro* findings with the history and physical evidences of mastitis in the respective animals, (3) a comparison of the strains isolated from the various quarters of one udder, and (4) a study of possible group relationships of the various strains.

The *in vitro* tests used were those suggested by Chapman *et al.* (1933–1938). Accordingly, 41 of the 114 strains showed the reported evidences of pathogenicity. Eleven of these 41 were from cows suffering from mastitis and the remaining 30 strains were

isolated from cows having no known history or evidences of the disease. Three *similar strains* were isolated from different quarters of the udder from each of two cows, and five similar strains from each of five cows.

On the basis of the tests used, this collection of cultures was a rather heterogeneous one as they were divided into one group of 12 similar strains, one group of four, one of nine, and nine of two strains each. The remaining 53 strains were dissimilar.

TESTING THE EFFICIENCY OF RHIZOBIUM MELILOTI. *J. T. Kroulik*, Department of Bacteriology, Kansas State College, Manhattan, Kansas.

The following method proved satisfactory for testing the nodule forming and nitrogen fixing efficiency of *Rhizobium meliloti* in a greenhouse thoroughly impregnated with strains of the same organism. 600 grams of thoroughly washed Ottawa sand were placed in 65 x 500 mm. Pyrex test tubes. A nutrient solution, containing $CaCO_3$, was added in quantity equivalent to that retained against gravity less 15 ml., th tubes plugged with cotton and sterilized. varied from 6.9% to 11.2% and for nitrogen fixed from 5.5% to 16%. The roots are easily freed of sand and the number and other characteristics of the nodules readily studied.

STUDIES ON E. COLI-MUTABILE. *W. A. Tanner*, Bacteriology Department, K.S.C., Manhattan, Kansas.

Escherichia coli-mutabile has been isolated and a rapid lactose-fermenting variant obtained. The mother culture, or slow fermenting organism, is referred to as the "W" strain, while its rapid fermenting variant is designated as the "R" strain.

Physical and chemical studies have been made on these strains in an effort to find the primary cause of the latent action of lactose. Chemical analyses of the products of lactose fermentation yielded carbon dioxide, lactic acid or succinic acid, acetic acid and ethyl alcohol as the final end products. It was found that although the slow fermenting strain ("W") utilized some lactose from the beginning, it did not utilize an appreciable amount for 5-10 days. At the end of 10 days, both strains had fermented equal amounts

burg apparatus were used in the study of respiratory enzymes on "resting" bacteria of both culture types. That is, the organisms were washed and aerated and did not proliferate to any appreciable extent. The results obtained showed a direct correlation between dehydrogenase and oxidase activities in the instances where glucose, sodium lactate, sodium succinate, sodium pyruvate, sodium fumarate, and sodium citrate were used as hydrogen-donators. In the cases of lactose and sodium acetate, the substratum was found to be more active as a hydrogen-donator when oxygen acted as the acceptor than when methylene-blue was used for that purpose.

Catalase tests were run, using an original technique that required the use of the Warburg apparatus. It was made evident that both "R" and "W" forms of *E. coli-mutabile* were prolific catalase producers.

THE UTILIZATION OF CERTAIN CARBOHYDRATES AND SUGAR DERIVATIVES BY RHIZOBIA AND CLOSELY RELATED BACTERIA. *C. E. Georgi and J. M. Ettinger*, Dept. of Bacteriology, University of Nebraska, Lincoln, Nebraska.

The ability of twelve Rhizobia (representing six cross-inoculation groups), *Bacterium radiobacter*, and *Azotobacter chroococcum*, to utilize d-glucose, d-fructose, d-xylose, melibiose, cellobiose, melezitose, dextrin, amygdalin, inulin, and inositol has been studied. Carbohydrates in these media were sterilized by filtration in order to compare these findings with one reported in which the medium was rendered sterile by autoclaving.

A group of sugar derivatives, calcium gluconate, sodium salts of d-gluconic, d-mannonic, l-mannonic, d-galactonic, and mucic acids as well as di-sodium saccharate have been investigated with respect to their availability to the previously mentioned bacteria.

Practically all of the carbohydrates and sugar derivatives studied were utilized by the Rhizobia, *B. radiobacter*, and *A. chroococcum*. Efficiency (in terms of nitrogen fixation) of the root nodule bacteria could not be differentiated on the basis of fermentation characteristics.

Attention is called to two notable exceptions however. Rapid acid production of *B. radiobacter* on sodium d-gluconate and on sodium l-mannonate gives promise of differentiating this common nodule inhabitant from the Rhizobia. Failure of *A. chroococcum* to grow on media containing calcium d-gluconate in the absence of added calcium carbonate is being further investigated.

Changes in reaction of the media observed during the incubation period are discussed.

DIFFERENTIAL BLOOD PICTURE AND TOTAL COUNT STUDIES ON NORMAL AND TRICHINAE-INFECTED ALBINO RATS. *Edgar H. Beahm and Cornelia M. Downs*, Dept of Bacteriology, University of Kansas.

Studies on human trichinosis have shown that eosinophilia is so constantly associated with trichinosis that its presence may serve as an important factor in the diagnosis of the disease. Although the haematologic response of man to infections of *Trichinella spiralis* is quite well established, the same cannot be said of a number of laboratory animals that are used a great deal in experiments on this parasite.

Blood studies, both total and differential, on a series of 20 albino rats revealed that the total erythrocyte count was not altered following infec-

tion with *Trichinella spiralis*. The total leukocyte count underwent a great increase when infectious doses of 600–700 muscle-encysted trichinae larvae were eaten by the host; however if only 25–50 larvae were ingested the count changed very little. It was also found that doses between these two extremes brought about reactions in direct proportion to the severity of the infection.

The differential count is also dependent upon the number of trichinae ingested. If the infection is severe enough a relative and absolute neutrophilia appears. This takes place at the expense of the lymphocytes bringing about a relative lymphopenia. Within 3 or 4 days the lymphocytes begin to increase noticeably and a lymphocytosis results. The decrease in neutrophiles is due to the fact that a great many lymphocytes have been released into the blood stream.

The eosinophil count, which runs very low in normal animals, showed no appreciable change after infection; the same was true for the basophil and monocyte determinations.

STUDIES ON THE DISTRIBUTION OF MIGRATING TRICHINELLA LARVAE IN RATS. *Edgar H. Beahm and A. B. Leonard*, Dept. of Bacteriology and Dept. of Zoology, University of Kansas.

One hundred albino rats were infected by feeding trichinosed meat, and killed thereafter at intervals of four hours. Observations were made on excystation and maturation of worms in the intestine. There was no localization of the worms in specific regions of the intestine; many were found even in the caecum and colon. The maturing worms wandered freely into the intestinal mucosa, and induced a tenacious mucus exudation. Sexual differentiation was first seen at 32 hours, matured eggs at 100 hours and larvae in the females 24 hours later. Migrating larvae were first recovered from the peritoneal cavity six days after infection, and soon after from cardiac blood, the pleural cavity and observed in frozen sections of the lung. Larvae were found in small numbers in the portal circulation and also in the cerebro-spinal fluid.

The entire intestinal tract, liver, spleen, kidney, tongue, brain, heart and samples of muscle were preserved for sectioning. In spite of the ubiquity of the migrating larvae in the circulatory system and the body cavities careful study has thus far failed to reveal their presence in the lymphatic system, the kidney, the liver, the spleen or the heart. Migration of the larvae into the muscle began at about the 15th day.

Pathological histology of the gut was studied.

OBSERVATIONS ON THE IDE PRECIPITATION TEST FOR SYPHILIS. *Clarence L. Brumback*, University of Kansas.

A comparative study of the Ide test with the Kolmer- Wassermann, Kahn, and Kline tests led to the conclusion that the Ide was somewhat less specific and considerably less sensitive than the other three tests. However, its simplicity, ease of reading, and shortening of the time required for testing, together with the possibility of its application to whole blood suggest its use as a supplementary clinical test in the diagnosis of syphilis.

The test showed an agreement of 89.5% with the Kolmer, 89.9% with the Kahn, and 92.9% with the Kline, considering the strongly positive (4+ and 3+) reactions obtained on 665 serums. On 1129 serums from persons clinically negative and giving no his-

tory of syphilis, 2 positive reactions were obtained with the Ide. The other tests were negative on these serums. The Kolmer gave 1223 negative reactions on a total of 1467 serums as compared with 1241 obtained with the Ide. On 680 serums tested with the Kahn, Kline, and Ide tests, 425 negative reactions were shown by the Kahn, 435 by the Kline, and 442 by the Ide.

AGGLUTININS FOR B. ABORTUS AND RELATED ORGANISMS IN BOVINE SERUM. *Cornelia M. Downs and Glenn C. Bond*, University of Kansas, Lawrence.

The studies here reported are concerned with the relation of antibodies agglutinating members of the *Brucella* group and *Pasteurella tularensis* and related organisms. Human serum from undulant fever cases occasionally agglutinates tularense and serum from cases of tularemia not uncommonly agglutinates abortus organisms.

In one series 102 bovine sera furnished through the kindness of Dr. L. D. Bushnell were set up against the following antigens:

1. Human strain *Brucella abortus*.
2. Bovine strain *Brucella abortus*.
3. Suis strain *Brucella abortus*.
4. Three strains of *Alcaligenes bronchisepticus*.
5. Five strains of *Pasteurella boviseptica*.
6. Three strains of *Pasteurella tularensis*.

A table, not presented here, showed the distribution of positive agglutination reactions. The significance of agglutinins for *A. bronchisepticus* and *P. boviseptica* is unknown. The infrequency with which these serums show agglutinins for *P. tularensis* is in line with the usual report that few human undulant fever serums exhibit agglutinins for *P. tularensis*. In a short series of 15 undulant fever serums furnished by the State Board of Health Laboratories, one only agglutinated *P. tularensis*. However out of 15 tularemia serums, 6 agglutinated *B. abortus*, and out of 21 tularense immune rabbit serums 10 agglutinated *B. abortus* in low dilution.

The zoning is more marked in bovine serums than in human or rabbit serums and the occurrence of middle zones in 3 serums with the 844 antigen is particularly noted. The zoning phenomenon evidently has to do with both the serum and the antigens used.

A STATISTICAL ANALYSIS OF THE INCIDENCE OF CONTAGIOUS DISEASES IN THE STUDENT NURSE POPULATION. *J. D. LeMar*, University of Nebraska College of Medicine, Omaha, Nebraska.

THE RÔLE OF CARBON DIOXIDE IN THE RESAZURIN TEST. *Millard F. Gunderson and Hugh L. Templeton*, University of Nebraska College of Medicine and Roberts Dairy Company, Omaha.

Addition of carbon dioxide to a 0.05% aqueous stock solution of resazurin caused the formation of purple-black particles which could be filtered or centrifuged out of suspension. The supernatant liquid was red.

The precipitated material was quite insoluble in carbon-dioxide-saturated water, but dissolved rapidly in dilute alkali or water from carbon dioxide. The resulting blue solution behaved in the normal manner.

Concentration of the red component with simultaneous removal of the carbon dioxide gave a blue solution with a slightly red tinge which seemed identical with resazurin when used in milk.

Addition of approximately 1.5 grams

of carbon dioxide to 10 ml. of milk lowered the pH of the milk about 0.5 of a pH unit. Foaming was very pronounced in pasteurized milks, milks with high bacterial counts and alkaline milks.

Very small amounts of carbon dioxide will cause color changes in dilute aqueous solutions of 0.1 ml. of a resazurin stock solution in 10 ml. of water.

The use of carbon dioxide to speed up the resazurin test showed that while the first color change from blue to mauve was accelerated the total time for the change from blue to white was about the same as in the control samples.

Some possible explanation of the reactions involved are offered.

FERMENTATION OF SUGAR ACIDS. *Gordon B. Robbins and Keith H. Lewis,* Departments of Chemistry and Bacteriology, University of Nebraska, Lincoln, Nebraska.

The fermentation of twelve sugar acids was studied with respect to thirty-two strains of bacteria belonging to eleven genera. Seven of the compounds had not been used previously in such studies.

Comparison of the aldoses, alcohols, and aldonic acids in the glucose, mannose, and galactose series supports the conclusions of previous workers that the aldoses are more readily fermented than either the alcohols or the acids.

Failure of l-rhamnonic acid to be fermented by species which attack l-rhamnose indicates that the fermentability of either aldose or acid cannot be predicted by the reactions of the other compound.

Although d-gluconic and d-mannonic acids were fermented by many organisms, their optical antipodes were not attacked.

The results suggest that further investigation may lead to data of value for differentiation of closely related bacteria, and to a better understanding of the relationship between configuration and fermentability.

FACTORS LIMITING BACTERIAL GROWTH

V. Fractional Sedimentation of Shigella

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Our previous experiments with *Escherichia coli* have led to the conclusion that the rate of accretion in nutrient broth during the early hours of growth is constant, and that the differences in initial rate of multiplication of transplants from younger and older cultures is attributable to differences in average size (Hershey, 1939). It follows that if smaller and larger individuals could be isolated from a single culture, these should show differences in rate of multiplication, i.e., the larger cells should undergo fission sooner than the smaller, other conditions being equal, when transferred to nutrient broth. Furthermore, knowing the rate of growth and the maximal adult size, the average time required for cells of given size to divide should be predictable from extremely simple laws, independently of the "physiologic state" of the parent culture.

In order to subject these predictions to experimental test, we have attempted to separate bacteria of larger and smaller size from day-old aerated broth cultures by fractional centrifugalization. By the methods employed, differences in average size of 10 to 20 per cent were obtained between the more rapidly and more slowly sedimenting fractions of *Shigella*. With *E. coli* no significant differences were found (table 2). Accompanying the differences in size, differences in latency of cell division of 6 to 16 minutes were observed, the shorter times being associated with larger size (fig. 1 and table 2). No appreciable differences were seen with *E. coli*, experiments with the latter organism serv-

ing as excellent controls. Failure to obtain separation of size with the motile species we attribute to interference by flagella.

TABLE 1

Rate of accretion and average adult size of E. coli and Shigella flexneri

ORGANISM	INCUBATION PERIOD	RATE OF ACCRETION†	ADULT SIZE‡
	hours		
*E. coli**		3.1	16.1
Shigella flexneri	2.5	2.5	16.5
	2.5	2.4	14.3
	3.0	2.6	13.0
	3.0	2.5	9.0

* Average of numerous measurements.
† Doublings per hour.
‡ O_2 consumed, $mm.^3 \times 10^{-7}$ per cell hour in broth.

TABLE 2

Average "size," and latent period in subculture, of cell-fractions obtained by centrifugalization

DATE OF EXPERIMENT	ORGANISM USED	FRACTION	SIZE*	L OBS.†	L OBS.‡	L CALC.§
				hours	*hours*	*hours*
2/27/39	*E. coli*	Sediment	1.40	1.33	1.32	1.28
		Supernate	1.53	1.33	1.25	1.24
3/ 7/39	*Shigella flexneri*	Sediment	1.17	1.56	1.61	1.58
		Supernate	0.98	1.83	1.87	1.67
3/14/39	*Shigella flexneri*	Sediment	0.93	1.68	1.70	1.71
		Supernate	0.85	1.77	1.78	1.76
3/21/39¶	*Shigella flexneri*	Sediment	2.10	1.20	1.37	1.23
		Supernate	1.90	1.33	1.48	1.29

* Oxygen consumed, $mm^3 \times 10^{-7}$/cell hour at zero time in broth.
† Time required for 50 per cent increase in numbers.
‡ Average of $L = t - nG$ observed for the first three generations; t = midpoint of successive cell divisions; n = number of corresponding generation; G = reciprocal of rate of accretion.
§ Assumes growth at constant rate to constant average size preceding cell division.
¶ 18 hour agar slopes as starting material. Day old aerated broth cultures in all other experiments.

There was no significant difference in rate of accretion between the two fractions with either organism.

Although the differences are small, no attempt has been made to subject the data to statistical analysis. Their validity rests not so much on the question of random error, as on interpretation. Our methods do not distinguish between bacteria existing as larger and smaller individuals, and larger and smaller groups of cells. An attempt was made to test the possibility that the rapidly-sedimenting fraction contained cells in greater aggrega-

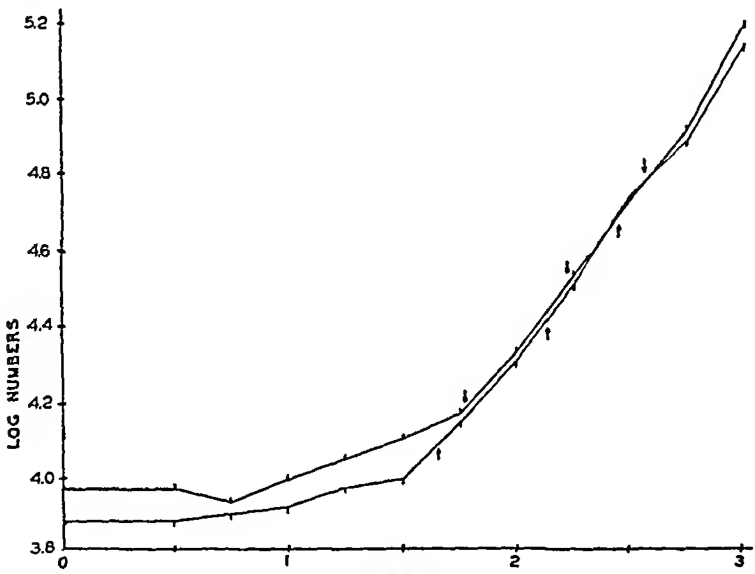

FIG. 1. MEASUREMENT OF LATENT PERIOD OF SLOWLY AND RAPIDLY SEDIMENTING FRACTIONS OF SHIGELLA

Experiment 3/14/39 (table 2). Upper curve supernate, lower curve sediment fraction. Arrows indicate the mid-points of successive generations.

tion by making use of the fact that this circumstance should lead to an abnormally high resistance to heat as measured by the viable count. No differences were observed in this respect between the top and bottom fractions, but the actual difference in size is probably too small to permit evaluation by this method. Microscopic examination of fresh preparations revealed very little aggregation of either fraction. Fixed smears could not be used for comparative counts because of salt-agglutination which

occurred as the supensions dried. Final resort was made to the special precautions taken to homogenize the preparations, and to the probability that repeated counts made during the latent period of the individual cultures would reveal any discrepancies resulting from dissociation of aggregates.

These experiments were undertaken with a broader purpose in view. It was hoped by this method to obtain bacterial fractions in the same "physiologic state" but differing sufficiently in size to enable comparison to be made of relative resistance to injurious influences, and of other properties differing between bacteria from young and old cultures. This hope was not realized. Nor are the differences entirely satisfactory, quantitatively, with respect to growth properties. Nevertheless, since they are rigorously consistent, both with themselves and with subsequent data of another kind, the experiments will be described in detail.

METHODS

Pure-line strains of smooth cultures were used, never more than three transplants removed from uniform lots of desiccated culture. Nutrient broth was seeded with approximately 10^6 bacteria per ml., and vigorously aerated at 37°C. for a full day. Cultures prepared in this manner are metabolically at rest, due to exhaustion of nutrient material (Hershey and Bronfenbrenner, 1937), so that there is no possibility of bacteria entering upon a new growth cycle during subsequent fractionation. The culture was usually subjected to a preliminary separation at about 2,000 R.P.M. (mean radius 20 cm.) for 20 minutes. The sediment so obtained was resuspended in 2 ml. saline, aspirated violently 100 times with a pipette carrying a strong rubber bulb, diluted further to about half the original volume of the culture, and centrifugalized for 3 to 5 minutes. This procedure was repeated 2 or 3 times, each time discarding the supernate, until the final sediment contained about 10^{10} organisms.

The supernate from the initial centrifugalization was similarly fractionated, except that the more slowly sedimenting fraction was recovered each time, an angle centrifuge being used for this purpose. The whole procedure was carried out in such a way that

the two fractions were handled simultaneously, receiving the same exposure to saline, before use. They were finally taken up in salt solution, homogenized as before, and diluted to the same turbidity (approximately 10^9 per ml.). Each fraction comprised roughly 4 per cent of the starting material.

Size and growth rates were measured manometrically (Hershey, 1939). The nephelometric method was unsuitable, since it yielded fictitiously large values for size with the bottom fractions, because of the concentration there of the slight amount of non-bacterial sediment present in the original culture. Presumably nitrogen determinations would be misleading for the same reason. The growth rate was obtained by measurement, after 2 to 3 hours incubation, of oxygen consumption of aliquots of 1:100 dilutions in broth of the original suspension.

Simultaneously with the above measurements, and immediately after completing the fractionation, cultures were seeded with each of the two fractions for measurement of latent period. These consisted of bottles containing 100 ml. of broth at 37°C. which received, for convenience in counting, approximately 10,000 bacteria per ml. Carefully timed at about 15-minute intervals, 1:100 dilutions were made in saline, vigorously shaken 50 times, and plated in five 1 ml. amounts. Plating was continued throughout three or four generations.

The initial numbers of bacteria were ascertained by averaging the counts made prior to a significant (greater than 10 per cent) and sustained increase in numbers, usually the three or four counts made during the first hour. The mean latent period, i.e., the time required for 50 per cent of the original organisms to divide, was read off from the graph of the observed counts. In order to minimize the effect of errors in individual counts, a second value was obtained by locating on the graph the time corresponding with a 50 per cent increase during the second generation, and subtracting the generating time, calculated from growth rate, from this. Similarly, the median of the third generation was found, from which two generation times were subtracted, and the three results were averaged. This average, as well as the values for the first generation, are shown in table 2.

The agreement between them, usually very close, provides a measure of the consistency of the data. An example of the method is shown in the figure.

The latent period required by theory, also shown in table 2, was calculated from the observed size, and from the data of table 1, using an equation describing growth at constant rate. The agreement between calculated and observed values within the individual experiments must be regarded as somewhat fortuitous, inasmuch as the differences in size recorded are nearly within limits of experimental error. For this reason, the method of calculation will be left for another publication, which will include observations of a more general character.

The four experiments summarized in table 2 were performed consecutively. Included are one of the several negative trials with *E. coli,* and all the experiments carried out with *Shigella flexneri.*

SUMMARY

By centrifugalization, fractions were obtained from cultures of *Shigella flexneri*, but not of *Escherichia coli*, showing small differences in average size of cells. The larger organisms exhibited a shorter latent period in nutrient broth than the smaller, the differences being of the order of magnitude required by theory.

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NONMOTILE VARIANTS OF BACILLUS ALVEI[1]

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The ability of *Bacillus alvei* to form migrating colonies upon the surfaces of solid media free from excessive moisture was reported previously by Smith and Clark (1938).[2] A similar property has been noted by Roberts (1935) for *Bacillus rotans*. Recently Shinn (1938), in a cinematographic study of colony motility of *B. alvei*, remarked that it was surprising that such motility had been so long overlooked, and questioned whether the phenomenon applied to *B. alvei* or to a bacterium described as "*Bacillus helixoides*" by Muto (1904).

Following our original demonstration of colony motility in *B. alvei*, additional cultures, isolated from foulbrood of bees and labelled *B. alvei* or *Bacillus para-alvei* have been received which likewise show colony migrations. All of our own isolates from soil, considered identical with named cultures of *B. alvei*, have shown motile colonies. Reports of earlier workers (Cheshire and Cheyne, 1885; Harrison, 1900; White, 1906; Tarr, 1935) upon *B. alvei* contain descriptions of its growth upon solid media which suggest that the phenomenon of colony motility had occurred, even though the actual migration had not been observed. Such references, together with the motility of authentic cultures, have led to the present study to determine whether colonies of *B. alvei* always show motility under suitable conditions.

[1] Investigations conducted coöperatively by the Division of Soil Microbiology, Bureau of Plant Industry, U. S. Department of Agriculture, and the Kansas Experiment Station. Contribution No. 177 of the Department of Bacteriology, Kansas State College of Agriculture and Applied Science.

[2] The author is indebted to N. R. Smith for valuable suggestions offered in the completion of the present work.

ISOLATION OF COLONY VARIANTS

The culture of *B. alvei* employed in these studies was obtained from J. I. Hambleton, Bureau of Entomology and Plant Quarantine, Beltsville, Md. This culture, found morphologically and culturally identical with a second culture from Hambleton and with a culture of *B. alvei* obtained from A. G. Lochhead, Central Experiment Farm, Ottawa, has been under observation for a period of 20 months for variations in its colony motility.

Isolation of variants from this culture was accomplished by streaking single lines of inoculation across Petri dish surfaces of sterile nutrient agar. After incubations of from 1 to 4 days at room temperatures, such surfaces were covered by migrating colonies. Occasional colonies could be observed which exhibited more elevated or more mucoid appearances. Upon re-streaking these to subsequent plates, it was possible to obtain, after a few subcultures, plates showing colony formation limited to the line of inoculation. In further subculturing, such daughter strains quite frequently showed motile out-growths at one or more points along the line of inoculation, and it was only after continued selection of nonmotile colonies that strains were established which could be carried for successive subcultures on solid agar without showing motile habit of growth.

PROPERTIES OF NONMOTILE VARIANTS

The nonmotile variants showed the cultural, tinctorial and physiological responses of the motile types except in case of the following characteristics:

Appearance of growth on solid agar

Nonmotile strains produced round, raised, convex colonies on solid agar, with growth limited to the line or point of inoculation. On the other hand, motile strains produced many irregular colonies, frequently vermiform or helicoid, and growth was not limited to the line or point of inoculation.

Motility

Hanging drop mounts prepared from nonmotile colonies did not show the presence of motile organisms; in contrast, active motility was exhibited by cells from motile colonies.

Flagella

From motile colonies, peritrichic flagellation of cells could be demonstrated readily; on the contrary, efforts to stain flagella on cells obtained from nonmotile colonies failed repeatedly, although areas of precipitation or of light staining were obtained about the individual cells.

Capsules

Using the India ink method of demonstration, cells from nonmotile colonies showed the presence of large capsular areas, in contrast to the lack of such distinct encapsulation in the motile strains.

Glucose broth

Nonmotile variants produced a granular type of growth, with sediment, whereas motile variants produced an early uniform turbidity following inoculation.

In all physiological responses the two types were found similar. Differences were not observed in sizes of cells or spores, or in numbers of spore-bearing rods or free spores in cultures of comparable ages. In stained smears prepared from suitably aged nutrient agar cultures of either the motile or nonmotile type, long rows of cylindrical spores, with the long axes of the spores lying parallel, were observed. The formation of such rows of spores by *B. alvei* has been noted frequently by earlier workers (McCray, 1917; Lochhead, 1928; Tarr, 1935), and is considered almost diagnostic of this species.

STABILITY OF NONMOTILE VARIANTS

The nonmotile habit of growth was difficult to establish, but once obtained, nonmotile variants were relatively stable on solid

media. Such variants could also be passed through broth for limited periods without loss of their characteristic nonmotile form of growth. Suspensions of their spores boiled for from 5 to 10 minutes and plated again gave rise to nonmotile colonies. On the other hand, after several transfers through broth media, after aging of a single culture in broth for several days, or occasionally spontaneously, nonmotile variants would again show the motile habit of growth. In contrast, motile strains were never observed to become spontaneously nonmotile, either after boiling of spores, after subculturing on various common laboratory media, or after incubations of inoculated plates under different conditions of light, temperature or position.

DISCUSSION

These studies upon *B. alvei* have shown that, even when conditions suitable for colony migration exist, nonmotility of its colonies may occur. The tinctorial and cultural evidence indicates that lack of motility in selected cultures is associated with an excessive amount of capsular or extracellular material. The production of variants unusually well endowed with capsular material raises again the question of the importance of extracellular material in the phenomenon of colony migration generally. Roberts (1935) has suggested that the migration of colonies of *B. rotans* may result from the action of flagella within a watery secretion produced by the cells themselves. A limited amount of cohesive extracellular material could undoubtedly contribute to the unity exhibited by a number of cells grouped into a mobile unit.

The occurrence of long rows of spores in stained preparations from nonmotile cultures, identical to rows of spores produced by motile cultures, is of especial interest. In direct microscopic observation of motile colonies, the individual bacilli are observed to lie parallel to one another, an arrangement significant in the motility of a group of cells as a unit, and it is not surprising that in stained preparations spores are observed lying in lateral rows. The appearance of similar rows of spores in stains from nonmotile colonies indicates an orientation of cells, even though actual migration does not occur.

The greater stability of the motile over the nonmotile type of growth makes it plausible to assume that earlier descriptions of plate growths of *B. alvei* were based on cultures showing motility of colonies, even though the actual phenomenon of migration was not observed. Cheshire and Cheyne (1885) noted that on gelatin plates outgrowths from the line of inoculation occurred, that such outgrowths might grow round so as to form a circle, and that from such circles other fresh circles might be formed. Harrison (1900) observed spreading and repeated branching of growth on agar plates, and believed the seaweed appearance "distinctively characteristic, and as the growth is very rapid, this method commends itself for making a quick diagnosis. . ." That White (1906) possibly encountered colony motility is indicated by his observation that colonies were often "surrounded by numerous smaller but similar growths." More recently, Tarr (1935) has noted that on agar (dried), "there is marked spreading, the growth appearing as a mass of colonies over the surface of the medium."

These references suggest that the spreading habit of growth has long been considered characteristic of *B. alvei*. Some observations on variability in this species have also been noted. Lochhead (1928) reported an asporogenic habit of growth by *B. alvei* on certain sugar-containing media, and that the further aging of the non-sporulating rods on suitable media produced coccoid types, relatively stable in further culture. Burnside (1934) produced asporogenic cultures of *B. alvei* by repeated passages through broth, and noted that such cultures were nonmotile. Whether the sporogenic nonmotile variants which we have secured on ordinary nutrient agars are related in any manner to, or are intermediate between, the streptococcus-like types noted by Lochhead or the asporogenic nonmotile types noted by Burnside is not known.

Shinn (1938) has questioned whether *B. alvei* and *Bacillus helicoides* (the latter described by Muto in 1904 as *B. helixoides*, and amended to *B. helicoides* by Kitasato (Lehmann and Neumann, 1931)) are not "one and the same," noting that his strain (*B. alvei* A. T. C. C.) "would, on cursory examination, answer

best to Muto's description." Apparently Shinn did not fully consider the original description, because *B. helicoides* was described as a non-sporing gram-negative rod, failing to withstand heating to 60°C. for 10 minutes, and failing to attack gelatin or milk. Lehmann and Neumann (1931) have considered *B. helicoides* to be related to *Proteus vulgaris*; it should not be confused therefore with *B. alvei*. It has been noted previously by Smith and Clark (1938) that colony motility is not limited to the genus *Bacillus*; the colonies of a *Bacterium* isolated from the intestinal tract of an angle-worm showed motility.

Russ-Munzer (1938) has also reported aerobic gram-positive spore-bearing rods showing colony migrations. Her cultures have been identified by N. R. Smith (personal communication) as *B. alvei* and as *Bacillus circulans*.

SUMMARY

From a culture of *Bacillus alvei* showing motile colonies on dried agar surfaces, variant daughter strains which fail to show colony motility were obtained by selective picking. Such nonmotile variants showed reversion to the motile type when aged in glucose broth.

Lack of colony motility is associated with lack of demonstrable flagella, the presence of a large amount of extracellular or capsular material, and with a granular, rather than a turbid, type of growth in broth. Otherwise, the nonmotile variants were identical with the motile parent culture, even insofar as showing orientation of spores in long lateral rows.

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AN EXPERIMENTAL STUDY OF THE RELATION BETWEEN CONCENTRATION OF DISINFECTANTS AND TIME REQUIRED FOR DISINFECTION

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The mechanism of disinfection has been studied by a considerable number of investigators but most of the published work, and especially that concerning the relation between concentration of disinfectant and time required for disinfection, was done many years ago. It seemed worth while, therefore, to make a further study of this phase of the subject, with special reference to the possible practical utility of mathematical formulas.

An excellent discussion of the mechanism of disinfection, with reference to the literature, is contained in the treatise on Physiology and Biochemistry of Bacteria, by Buchanan and Fulmer (1930) and no attempt will be made here to review the literature.

In the present work the equation $C^n \cdot t$ = a constant (A) has been assumed to be the basic equation for the mechanism of disinfection. This equation was employed by Watson (1908) without details regarding its ultimate derivation. Buchanan and Fulmer have shown that it may be derived from the Guldberg-Waage law. Obviously its practical application presupposes strict uniformity in all experimental conditions and in all factors other than disinfectant concentration (C) and disinfection time (t).

The writer has determined the bactericidal efficiencies of several phenols and alcohols by a modified Rideal-Walker technic and from the experimental data thus obtained has calculated values of the concentration exponent (n) for these disinfectants. Calculation is facilitated by expression of the equation $C^n \cdot t = A$ in

the logarithmic form: $n \log C + \log t = \log A$. When disinfection times are determined for a series of disinfectant concentrations and the results are expressed in the equations: $n \log C_1 = \log A - \log t_1$, $n \log C_2 = \log A - \log t_2$, etc., successive pairs of equations may be combined by subtraction to yield the derived equations:

$$n_1 = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}, \quad n_2 = \frac{\log t_3 - \log t_2}{\log C_2 - \log C_3}, \quad \text{etc.}$$

from which an average value of n may be determined for the series.

The average value of n in such a series may also be determined by the graphical method employed by Watson. When the successive values for log C and log t are plotted against each other the results should lie in an approximately straight line. The value of n may then be calculated by the equation:

$$n = \frac{y_2 - y_1}{x_1 - x_2}$$

after selection of two suitable points (x_1, y_1) and (x_2, y_2) on the straight line drawn under guidance of the plotted points representing the individual tests. Both the above methods were employed in the present work and data on the agreement between the results will be presented. After the average value of n for the series had been obtained either by the graphical method or by computation, values of log A were obtained by substitution in the equation:

$$n \log C + \log t = \log A$$

and an average value of log A was determined for the series.

EXPERIMENTAL PROCEDURE

Bactericidal efficiency was determined by the Rideal-Walker technique, modified as follows: Beef-infusion broth adjusted to pH 7 was used instead of R-W standard broth; instead of a standardized dropping pipet a 1-ml. pipet graduated into tenths was used to measure the 0.5 ml. of culture required for the test; the usual medication temperature was 20°C. instead of 15° to 18°; dilutions were made in sterile Erlenmeyer flasks instead of

in cylinders; fixed amounts of the stock solution of disinfectant were added to varying amounts of distilled water, all measured with sterile standardized pipets; the time of exposure was extended beyond 15 minutes, subcultures being made at 2.5-minute intervals from 2.5 to 30 minutes, at 5-minute intervals from 30 minutes to 80 minutes, and at 10-minute intervals beyond 80 minutes; *Staphylococcus aureus* was used as a test organism in addition to *Eberthella typhosa.*

EXPERIMENTAL AND CALCULATED RESULTS

Experimental results with phenol as the disinfectant and *Staphylococcus aureus* as the test organism are shown in table 1,

TABLE 1

Bactericidal efficiency of phenol against Staphylococcus aureus

DILUTION	C (PARTS IN 1000)	t	LOG C	LOG t	LOG C_1 −LOG C_2 ETC.	LOG t_2 −LOG t_1 ETC.	n	LOG A
		minutes						
1:55	18.1	5	1.25768	.69897				7.87
1:60	16.6	7.5	1.22011	.87506	.03757	.17609	4.69	7.83
1:65	15.4	15	1.18752	1.17609	.03259	.30103	9.24	7.94
1:70	14.3	20	1.15534	1.30103	.03218	.12494	3.88	7.88
1:75	13.33	30	1.12385	1.47712	.03149	.17609	5.59	7.88
1:80	12.5	40	1.09691	1.60206	.02694	.12494	4.64	7.85
1:85	11.7	55	1.06819	1.74036	.02872	.13830	4.81	7.83
1:90	11.1	80	1.04532	1.90309	.02287	.16273	7.11	7.86
Average values							5.7	7.87

Experiment conducted at 20°C.

together with the derived values of n and log A. All calculations of the value of log A were based upon the average value of n = 5.7. The same experimental data are presented in Figure 1, and there lead to the average value, n = 5.75.

The resistance of test cultures is prone to vary in spite of efforts to maintain constancy in all experimental conditions. The effect of such variations is indicated by the data shown in table 2, derived from two experiments conducted on different days with phenol as the disinfectant and *Eberthella typhosa* as the test organism.

In 16 similar experiments with phenol and *Eberthella typhosa* the derived average values of n varied between 6.8 and 9.0 with a general average of 7.3. Likewise in 24 experiments with phenol as the disinfectant and *Staphylococcus aureus* as the test organism the derived average values of n varied between 4.6 and 6.6 with a general average of 5.9. However, 15 of the 16 experiments with *Eberthella typhosa* yielded values of n between 6.8 and 7.7 and 20 of the 24 experiments with *Staphylococcus aureus* yielded values of n between 5.4 and 6.6. It seems, therefore, that under the experimental conditions herein described the results of any

TABLE 2

Bactericidal efficiency of phenol against Eberthella typhosa

DILUTION	C (PARTS IN 1000)	EXPERIMENT 1		EXPERIMENT 2	
		t	n	t	n
		minutes		*minutes*	
1:65	15.4	5			
1:70	14.3	10	9.35	5	
1:75	13.3	15	5.59	7½	5.59
1:80	12.5	30	11.17	12½	8.23
1:85	11.7	50	8.76	22½	8.88
1:90	11.1			30	5.46
Average value of n			8.96		7.04

Experiments conducted at 20°C.

two comparable tests may be expected to vary by not more than 20 per cent. In a series of similar experiments results may be expected to come within 10 per cent of the general average for the series.

In partial confirmation of this estimate may be presented some additional results obtained in two experiments with phenol as the disinfectant and *Staphylococcus aureus* as the test organism. In these experiments the observed disinfection times for a 1:100 dilution of phenol were 150 and 180 minutes, respectively. The time calculated from the values of n and log A shown in table 1 would be 148 minutes but if the value, $n = 5.9$, the average for all the experiments, is substituted for $n = 5.7$ the calculated time

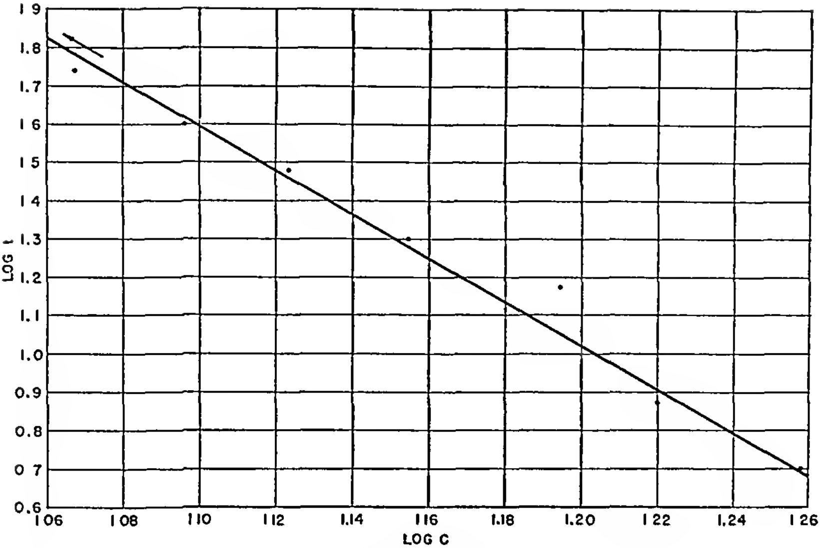

Fig. 1. Bactericidal Efficiency of Phenol Against Staphylococcus aureus, $n = 5.75$

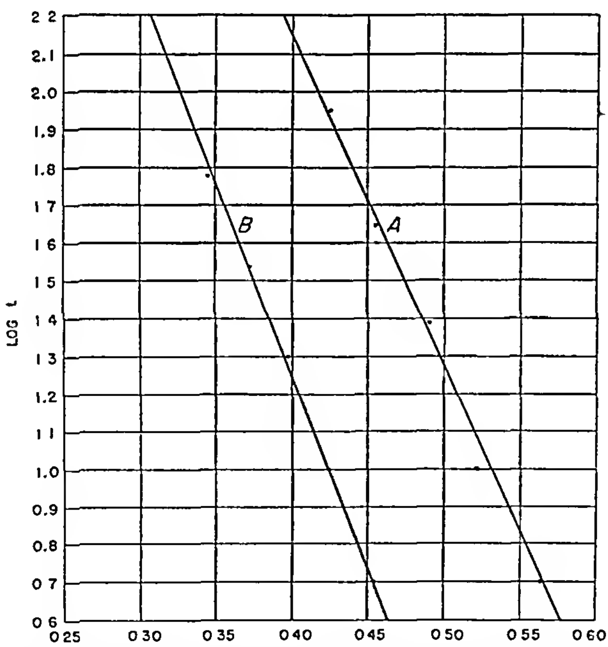

Fig. 2. Bactericidal Efficiency of Orthobutylphenol Against Staphylococcus aureus and Eberthella typhosa A, S. aureus, $n = 8.8$; B, E. typhosa, $n = 10.2$

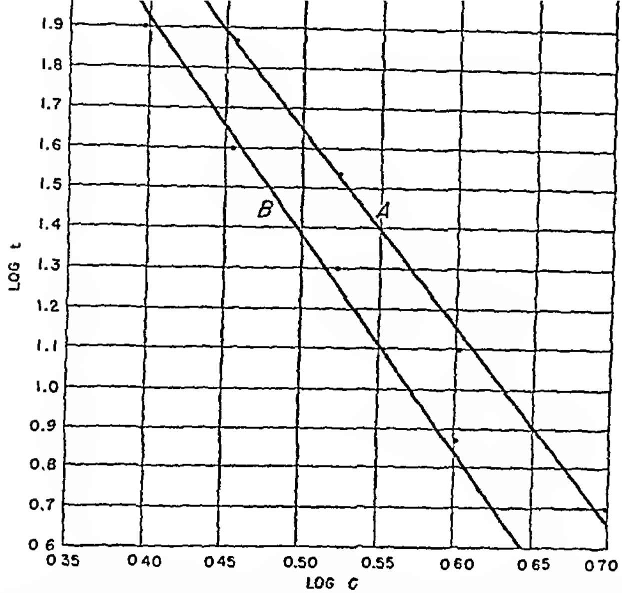

FIG. 3. BACTERICIDAL EFFICIENCY OF RESORCINOL AGAINST STAPHYLOCOCCUS AUREUS AND EBERTHELLA TYPHOSA, A, S. AUREUS, $n = 4.9$; B, E. TYPHOSA, $n = 5.45$

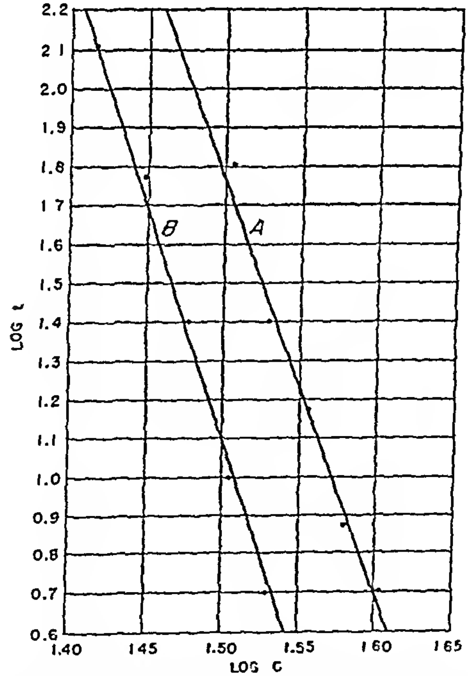

FIG. 4. BACTERICIDAL EFFICIENCY OF ETHYL ALCOHOL AGAINST STAPHYLOCOCCUS AUREUS AND EBERTHELLA TYPHOSA, A, S. AUREUS, $n = 10.7$; B, E. TYPHOSA, $n = 12$

TABLE 3

Bactericidal efficiencies of various disinfectants other than phenol

DISINFECTANT	EBERTHELLA TYPHOSA					STAPHYLOCOCCUS AUREUS				
	Dilution	C	t	n	log A	Dilution	C	t	n	log A
			minutes					*minutes*		
Orthocresol	1:150	6.66*	7.5		7.2	1:130	7.70*	5		7.3
	1:160	6.25	10.0	4.5	7.1	1:140	7.10	10	8.5	7.4
	1:170	5.88	15.0	6.6	7.1	1:150	6.66	20	10.8	7.5
	1:180	5.55	27.5	10.5	7.2	1:160	6.25	30	6.4	7.4
	1:190	5.26	40.0	8.8	7.1	1:170	5.90	40	5.0	7.4
	1:200	5.00	60.0	8.0	7.2	1:180	5.55	55	5.2	7.4
						1:190	5.26	90	9.2	7.3
Average values...				7.7	7.1				7.5	7.4
Paracresol	1:160	6.25*	5.0		7.1	1:130	7.70*	5		8.5
	1:170	5.88	7.5	6.6	7.0	1:140	7.14	10	9.3	8.5
	1:180	5.55	12.5	8.8	7.0	1:150	6.66	20	9.9	8.5
	1:190	5.26	20.0	8.8	7.1	1:160	6.25	30	6.4	8.5
	1:200	5.00	35.0	11.0	7.1	1:170	5.88	65	12.7	8.6
	1:210	4.76	50.0	7.1	7.1	1:180	5.55	90	5.6	8.5
	1:220	4.54	65.0	5.5	7.1					
Average values...				8.0	7.1				8.8	8.5
Orthobutyl-phenol	1:3500	2.85†	5		5.25	1:2750	3.66†	5		5.8
	1:3750	2.66	10	10.0	5.25	1:3000	3.33	10	7.3	5.7
	1:4000	2.50	20	11.1	5.28	1:3250	3.07	25	11.3	5.8
	1:4250	2.35	35	9.0	5.25	1:3500	2.85	45	7.7	5.7
	1:4500	2.22	60	9.5	5.24	1:3750	2.66	90	10.0	5.8
Average values				9.9	5.25				9.1	5.76
Parabutyl-phenol	1:4500	2.22†	5.0		3.9	1:3250	3.07†	5		5.3
	1:4750	2.10	7.5	7.3	3.9	1:3500	2.85	10	9.0	5.3
	1:5000	2.00	12.5	10.5	3.9	1:3750	2.66	15	5.9	5.2
	1:5250	1.90	17.5	6.5	3.8	1:4000	2.50	35	13.6	5.3
	1:5500	1.81	35.0	14.3	3.9	1:4250	2.35	75	12.3	5.4
	1:5750	1.73	50.0	7.9	3.9	1:4500	2.22	110	6.7	5.3
Average values				9.3	3.88				9.5	5.3
Resorcinol						1:20	50.0*	5.0		4.1
	1:25	40.0*	7.5		3.9	1:25	40.0	12.5	4.1	4.1
	1:30	33.3	20.0	5.3	3.9	1:30	33.3	35.0	5.6	4.1
	1:35	28.5	40.0	4.4	3.9	1:35	28.5	75.0	4.9	4.1
	1:40	25.0	80.0	5.3	3.9					
Average values..................				5.0	3.9				4.9	4.1

TABLE 3—*Concluded*

DISINFECTANT	EBERTHELLA TYPHOSA					STAPHYLOCOCCUS AUREUS				
	Dilution	C	t	n	log A	Dilution	C	t	n	log A
			minutes					*minutes*		
Normal propylresorcinol	1:225	4.44*	5.0		4.1	1:175	5.71*	7.5		4.9
	1:250	4.00	7.5	3.9	4.0	1:200	5.00	12.5	3.8	4.8
	1:275	3.63	12.5	5.3	4.1	1:225	4.44	22.5	5.0	4.8
	1:300	3.33	20.0	5.4	4.1	1:250	4.00	50.0	7.6	4.9
	1:325	3.07	30.0	5.0	4.1	1:275	3.63	80.0	4.8	4.9
	1:350	2.85	50.0	6.9	4.1					
Average values				5.3	4.08				5.3	4.86
Ethyl alcohol		34‡	5.0		18.0		40‡	5.0		19.0
		32	10.0	11.4	18.0		38	7.5	8.0	18.9
		30	27.5	12.1	18.1		36	15.0	12.8	18.9
		28	60.0	11.3	18.1		34	27.5	10.6	18.9
		26	130.0	10.4	18.1		32	65.0	14.2	19.0
Average values				11.3	18.06				11.4	18.94
Normal butyl alcohol		5.25‡	5.0		9.3		7.25‡	5.0		11.6
		5.00	10.0	14.2	9.4		7.00	7.5	11.5	11.6
		4.75	22.5	15.0	9.5		6.75	12.5	14.0	11.6
		4.50	45.0	12.8	9.5		6.50	20.0	12.4	11.6
		4.25	75.0	8.9	9.4		6.25	35.0	14.3	11.6
		4.00	130.0	9.1	9.3		6.00	55.0	11.1	11.6
Average values				12.0	9.4				12.7	11.6

* Parts in 1000.
† Parts in 10,000.
‡ Grams in 100 cc.

would be 166 minutes. Obviously, results can be predicted by the graphical method as an alternative to computation.

The results of experiments with a number of other disinfectants are shown in table 3 and, in part, in figures 2, 3 and 4. In each case the results are taken from a single experiment selected from a group of three or more.

The effect of soap in modifying germicidal efficiency is illustrated in figure 5. In order to facilitate comparison, data for Liquor Cresolis Saponatus are based on concentrations of the

contained cresols, which constituted approximately 50 per cent of the product. The two graphs cross at the point representing a cresol concentration of approximately 1:150. At lower concentrations, soap aids, and at higher concentrations, hinders, bactericidal efficiency as previously noted by the writer (Tilley and Schaffer, 1925).

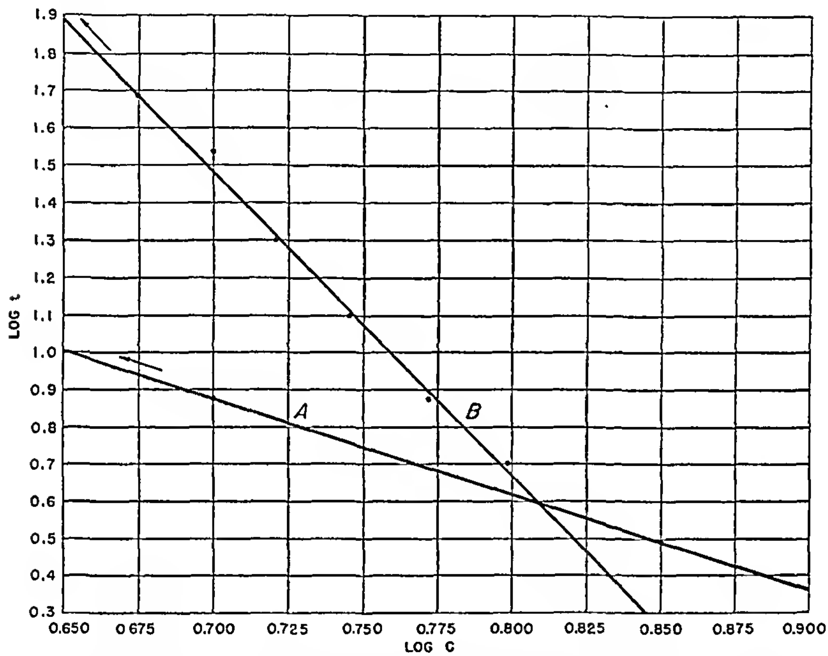

FIG. 5. EFFECT OF SOAP ON BACTERICIDAL EFFICIENCY OF CRESOL AGAINST EBERTHELLA TYPHOSA

A, Liquor Cresolis Saponatus on basis of cresol, $n = 2.57$; B, paracresol, $n = 8.2$.

In table 4 are presented values of n determined at several temperatures. These values are averages of two or more concordant results. In general they suggest very strongly that at ordinary temperatures values of n are characteristic for each disinfectant with each test organism. These characteristic values may be altered by temperatures of 30°C. or higher.

TABLE 4

Values of n determined at different temperatures

DISINFECTANT	TEST ORGANISM	10°C.	20°C.	30°C.	40°C.
Phenol	*E. typhosa*	7.9	7.5	5.9	5.2
	S. aureus	6.5	6.5	6.4	5.4
Orthocresol	*E. typhosa*	8.3	7.9	5.5	5.1
	S. aureus	7.8	7.6	8.1	6.8
Paracresol	*E. typhosa*	8.9	8.4	6.4	5.3
	S. aureus	8.2	8.7	9.4	7.0
O-Butylphenol	*E. typhosa*		9.2	7.2	
	S. aureus		8.5	9.1	
P-Butylphenol	*E. typhosa*		9.2	9.3	
	S. aureus		8.9	8.1	
Resorcinol	*E. typhosa*	4.6	5.1	5.2	
	S. aureus	4.4	5.0	6.2	
Ethyl alcohol	*E. typhosa*	12.7	11.4	8.8	
	S. aureus	11.4	11.1	8.5	
N Butyl alcohol	*E. typhosa*		11.9	10.3	
	S. aureus		11.8	10.1	

DISCUSSION

As Buchanan and Fulmer have pointed out, different types of disinfectant act upon bacterial protoplasm in different ways and it is not to be expected that the relation between concentration of disinfectant and time required for disinfection can be expressed by a single formula applicable to all types. However, it appears that these two factors are exponentially related over considerable ranges of concentrations and disinfection times in the case of many disinfectants and in such cases the derived mathematical values may be useful in evaluating or predicting bactericidal efficiency.

The mathematical formulas employed rigorously exclude any variation during comparable tests in any factors or conditions other than concentration of disinfectant and time required for

disinfection. It is impossible to control with mathematical precision such factors as the resistance and numbers of the bacteria in the test cultures and medication mixtures, and examination of the results presented in the tables and figures shows many irregularities due to unavoidable experimental errors. The effect of these errors is, however, minimized by averaging the results of each series of comparable tests and it is believed that average results obtained by the methods herein described may have some practical value.

The value, n, heretofore referred to as the concentration exponent is designated by some writers the coefficient of dilution, since it indicates the degree to which dilution lessens the rate of disinfection. For example, ethyl alcohol with a high value of n loses efficiency with dilution much more rapidly than phenol with a value of n about one half as great.

SUMMARY

The bactericidal efficiencies of phenol, orthocresol, paracresol, orthobutylphenol, parabutylphenol, resorcinol, n-propylresorcinol, ethyl alcohol and n-butyl alcohol against *Staphylococcus aureus* and *Eberthella typhosa* were determined by a modified Rideal-Walker technique. From the resulting experimental data values for the concentration exponent, n, were calculated by the formula:

$$n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

The value of n thus obtained was then substituted in the formula: $n \log C + \log t = \log A$ (a constant). Values of n and log A thus derived could be employed in this same equation to calculate either time or concentration when only one of these factors was known. The accuracy of results obtainable by the methods of testing employed is limited by unavoidable experimental errors but is believed to be sufficient to permit practical use of these mathematical formulas and derived values. Results of experiments conducted at different temperatures suggest that values of n

are essentially characteristic for each disinfectant with each test organism.

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STUDIES ON THE HEMOLYTIC STREPTOCOCCUS[1]

III. Polysaccharide and Protein Fractions Encountered in the Precipitation of Erythrogenic Toxin from Culture Filtrates

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In preliminary experiments designed to isolate pure erythrogenic toxin from streptococcal filtrates, extraneous products were encountered which complicated the separation. In this paper, five such products—a peptone polysaccharide, a uronic acid polysaccharide, a yellow "oil," syrupy proteins, and a "nucleoprotein" fraction—are to be discussed in sequence. The results are presented in some detail because they seem to be of value to the bacteriologist and the biochemist.

In this study, cultures of the NY5 strain of hemolytic streptococcus were made in three types of mediums which were used in sequence. Alcohol was employed to precipitate the toxin. The first medium consisted of Parke-Davis peptone and meat infusion. The small amount of toxin precipitated from the culture filtrate was associated with a peptone polysaccharide (Stock, 1937) that has been found to have no relationship to the toxin. The second type of medium was free from peptone polysaccharide. The small amount of toxin that was precipitated from such culture filtrates was mixed with two other substances, a uronic acid polysaccharide and a yellow "oil." In attempting to obtain all the toxin from the filtrates, syrupy protein products were precipitated. Consequently, a third type of medium was devised, one that did not contain readily precipitable polysac-

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charides or proteins. The "nucleoprotein" fraction was encountered with the first and second mediums.

I. A PEPTONE POLYSACCHARIDE

In our first attempts to isolate erythrogenic toxin from streptococcus culture filtrates of a Parke-Davis peptone-meat infusion medium, an interfering polysaccharide was found, as described in a preliminary communication (Stock, 1937). The source of the polysaccharide proved to be the peptone used in the medium.

TABLE 1

Comparison of peptone, pepsin, and pancreatin polysaccharides

PREPARATION	ASH	NITROGEN	REDUCING SUBSTANCE AFTER ACID HYDROLYSIS (CALCULATED AS GLUCOSE)	GLUCOSAMINE AFTER ACID HYDROLYSIS (CALCULATED AS N-ACETYL GLUCOSAMINE)	ACETYL	αD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*
Peptone polysaccharide (from culture filtrates)*..	1.5	7.0	60.0	37.5	9.0	0
Peptone polysaccharide*...	1.9	7.1	51.4	32.5		+16
Pepsin polysaccharide†....	0.0	6.16	70.7	33.5§	9.95	+16
Pancreatin polysaccharide‡.	1.9	4.0	71.1	22.5¶		−27

Our analyses calculated on ash-free and moisture-free basis.

* Derived from Parke-Davis Peptone. The small amount of uronic acid reported previously in the polysaccharide as obtained from culture filtrates (Stock, 1937) was not present in the polysaccharide isolated directly from the peptone.

† Analysis of Landsteiner and Chase (1936); Fairchild product.

‡ From Pfanstiehl product.

§ Recalculated from amino sugar value.

¶ Type of hexosamine not determined.

The analysis showed the presence of glucosamine, acetyl, galactose, and nitrogen. The analytical figures were almost identical with those of a polysaccharide from pepsin which had been reported by Landsteiner and Chase (1936) (see table 1) and had the properties of the blood Group A substance. The agreement between the analyses of the two polysaccharides indicated their probable identity, and it was suggested, therefore, that the origin of the peptone polysaccharide might be pepsin (stomach), because the latter is known to be one of the enzymic agents used in the manufacture of peptone.

A pancreatic polysaccharide

Because pancreatic preparations are also used as enzymic agents in peptone manufacture, we studied these as a possible source of peptone polysaccharide. We have found that trypsin preparations (Pancreatin, U.S.P. or "Trypsin," Pfanstiehl) contain a polysaccharide that is somewhat different, but that has components (nitrogen, galactose, hexosamine) which are common to the pepsin and peptone polysaccharides (see table 1). Therefore, one must consider the possibility that some peptones contain a polysaccharide derived from pancreas.

A considerable number of the commercial peptones tested by us contained a polysaccharide, whereas certain other peptones did not contain this substance.

It is a well known fact that certain peptones may support streptococcic growth without meat infusion (Ayers and Rupp, 1922; Rane and Wyman, 1937). Experiments indicate, when peptone is used without meat infusion, that only the group of peptones containing a polysaccharide have the capacity for supporting streptococcic growth. However, the polysaccharide may be removed from a quantity of growth-supporting peptone, and the residue serves as efficiently for a culture medium *per se* as the unaltered peptone. The polysaccharide, therefore, merely differentiates the two different groups of peptones, and is not an essential growth factor.

EXPERIMENTAL METHODS

Isolation of peptone polysaccharide and toxin from hemolytic streptococcus filtrates

The medium was a 1 per cent Parke-Davis peptone and beef-heart infusion which contained 0.05 per cent glucose and was sterilized by autoclaving at 15 pounds (115°C.) for 30 minutes. The stock cultures were maintained on blood (human) agar slants. The cultures were incubated 24 to 72 hours and were then filtered through a 10″ by 2″ Berkefeld V filter.

The essentials of the method of isolation, which is a modification of that of Ando, Kurauchi and Nishimura (1930) have been

outlined in a preliminary report (Stock, 1937). In brief, it consists of evaporation of the filtrate *in vacuo* at low temperature to 0.1 volume, precipitation of the toxin and polysaccharide with two volumes of alcohol, solution in water, removal of protein ("nucleoprotein") by acidification to pH 4.0, neutralization, followed by alcoholic reprecipitation, and finally dialysis in a Cellophane bag. These polysaccharide fractions had a golden-yellow color, to which reference will be made below.

Isolation of polysaccharide from peptone

A watery solution of Parke-Davis peptone was made and, with slight modifications, the procedures outlined for the isolation of the polysaccharide from filtrates was followed. Instead of neutralizing after acidification, the substance was precipitated directly with alcohol. Yield: 200 to 300 mgm. of light brownish powder per 10 grams of peptone. Further quantitative experiments on 10 gram samples of three other brands of peptone gave the following yields: Witte's peptone siccum, 105 mgm.; Difco's Proteose-peptone, 110 mgm.; Difco's Neo-peptone, 250 mgm. Qualitative chemical tests have indicated the presence of a peptone polysaccharide in the following additional peptones: Armour's peptonum siccum, Coleman and Bell's bacteriological, Fairchild Bros. and Foster, Pfanstiehl, Wilson, and Difco's Bacto-tryptose and Bacto-tryptone.

Peptones that did not contain a polysaccharide

Qualitative chemical tests have indicated the absence of a polysaccharide in single lots of the following peptones: Difco's Bacto-peptone and Bacto-protone, J. T. Baker's bacteriological, and Merck's "from meat-dried."

It is to be noted that most of the data on the various peptones were obtained from samples of single lots, and that other lots from the same manufacturer might give different results.

Chemical and physical analysis

The analysis refers to the polysaccharide from Parke-Davis peptone. Micro-analyses were made throughout. Due to the hygroscopic nature of the substance, all samples were dried

individually in small porcelain boats *in vacuo* in a Pregl block at 100°C., and weighed in micro "pigs." Total nitrogen was determined by Pregl's (1930) method using Goebel's (1932) modification of the distillation. The preparations were analyzed for ash by burning samples in a micro-muffle furnace. Acetyl was determined by Elek and Harte's(1936)method. The specific optical rotation was observed in a 1 dcm. tube in 0.5 to 1 per cent aqueous solutions. N-acetyl glucosamine, determined directly by Morgan and Elson's (1934) method gave a value of 13.2 per cent. Hydrolysis of the samples was accomplished with N/1 HCl for 2.5 hours. After neutralization to litmus with N/1 NaOH, aliquot parts were removed for the determination of glucosamine by Elson and Morgan's (1933) method and total reducing power by Hane's (1929) modification of the Hagedorn-Jensen method.

Typical crystals of glucosamine HCl were isolated by hydrolysis with N/1 HCl for two hours in a boiling water bath, followed by evaporation *in vacuo*, and repeated evaporation with methyl alcohol. The crystals were converted into typical needles of the anisal derivative (Bergmann and Zervas, 1931), the melting point of which was 164 to 165°C. Melting point of known samples of the anisal derivative 164 to 166°C.; mixed melting point 163°C.

Crystals of mucic acid were obtained by oxidation of the polysaccharide with HNO_3 according to Van der Haar (1920). The melting point of this compound was 209°C., whereas a known sample of mucic acid analyzed simultaneously melted at 212°C. The mixed melting point was 209°C.

The outstanding physical properties of the polysaccharide were listed in the earlier report (Stock, 1937).

Growth factors in peptones for the hemolytic streptococcus (NY5 strain)

To each solution of peptone to be tested, additions of 0.5 per cent NaCl and 0.05 per cent glucose were made, and the mediums sterilized in the autoclave at 115°C. for 30 minutes. Inoculations were made with a loop of organisms from a slant culture, and the ability to support growth after incubation from 24 to 72 hours was observed. One per cent solutions of each of the peptones

already listed were adjusted to pH 7.8 and each solution tested as just outlined.

The peptone polysaccharide was shown not to be an essential growth factor as follows: To a 10 per cent solution of Parke-Davis peptone (or Difco's Neo-peptone) two volumes of alcohol were added, and the precipitate, which contained the polysaccharide, was discarded. The alcohol was removed *in vacuo* from the supernatant fluid, the residue dissolved in water, adjusted to pH 7.8, and tested for growth-supporting capacity as above. Growth in this medium was as good as before the removal of the polysaccharide.

A polysaccharide in pancreas preparations

Extracts of pancreas preparations contain a polysaccharide as shown by the methods outlined above for peptone polysaccharide. Ten gram samples of commercial pancreatin, U. S. P., (Armour, Parke-Davis, Pfanstiehl, and Lilly) yielded 21 to 88 mgm. of clear, white polysaccharides. A small amount of yellowish polysaccharide was obtained from "Trypsin," Pfanstiehl. All polysaccharides gave strongly positive tests for hexosamine after acid hydrolysis. The type of hexosamine present was not determined. The polysaccharide from Pfanstiehl's pancreatin on oxidation with nitric acid according to Van der Haar (1920) gave crystals of mucic acid, which indicated the presence of galactose. The polysaccharide gave a negative uronic acid test (naphthoresorcinol).

II. URONIC ACID POLYSACCHARIDE PREPARATIONS

In our second attempts to isolate erythrogenic toxin, cultures were made in peptone-infusion mediums which were free from peptone polysaccharide. This type of medium was prepared in two ways. Either a peptone which does *not* contain the polysaccharide (*vide supra*) was incorporated with beef-heart infusion, or the residue of a peptone from which the polysaccharide has been removed (*vide supra*) was added to the meat infusion. The hemolytic streptococcus culture filtrates of this medium were precipitated with two volumes of alcohol, and the fractions

purified essentially as for the isolation of peptone polysaccharide and toxin from culture filtrates. The only difference was that following the acidification, the fractions were reprecipitated without neutralization.

Now, a different nitrogenous polysaccharide with an average yield of 50 mgm. per liter of filtrate was isolated together with small amounts of erythrogenic toxin. This substance was occasionally contaminated by small amounts of glycogen. Furthermore, at one stage in the alcoholic precipitation, a golden-yellow "oil" separated in 2 to 4 ml. quantities. This was recognized as the ingredient probably responsible for the above-mentioned golden color of the peptone polysaccharide isolated from hemolytic streptococcus filtrates.

TABLE 2

Comparison of uronic acid polysaccharides from various sources

PREPARATION*	ASH	NITROGEN	REDUCING SUBSTANCE AFTER ACID HYDROLYSIS (CALCULATED AS GLUCOSE)	GLUCOSAMINE AFTER ACID HYDROLYSIS (CALCULATED AS N-ACETYL GLUCOSAMINE)	URONIC ACID ANHYDRIDE	ACETYL	αD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*
Strain NY5-N1	1.7	6.5	66.0	24.2	28.7	6.0	−43
Strain NY5-Q1	3.8	9.0	44.7	22.2	+		−46
Strain Dick 2-Q1	6.1	6.6	36.2	21.5	+		−26
Strain H1-Q1	7.6	7.3	33.7	26.8	+		−34
Strain NY5 (Type 10 "Mucoid" polysaccharide)†	5.8	3.8	82.0	+	42.6	(11.0)	−86

Our analyses calculated on ash-free and moisture-free basis.

* Mediums employed for cultures: for preparation NY5-N1: beef heart infusion, and Parke-Davis Peptone freed from peptone polysaccharide; all Q-1 preparations: beef heart infusion and Bacto-peptone (polysaccharide free); Kendall et al Type 10 preparation: lean beef infusion and Bacto-peptone.

† Analysis of Kendall, Heidelberger, and Dawson (1937). Acetyl value from Type 5 preparation.

This polysaccharide gave a positive biuret test, a strongly positive uronic acid test (naphthoresorcinol), a negative direct Morgan and Elson test for N-acetyl glucosamine, and a positive test for hexosamine after acid hydrolysis. Quantitative analysis

of one preparation showed 6.5 per cent nitrogen, 6.0 per cent acetyl, 28.7 per cent uronic acid anhydride, and after acid hydrolysis, 24.2 per cent hexosamine (calc. as N-acetyl glucosamine) and 66.0 per cent reducing sugar (calc. as glucose). The preparations varied in their rotation of the plane of polarized light: negative rotations were usually encountered, but when glycogen was present, the rotations were to the right.

For comparison, two other strains of hemolytic streptococci, viz., scarlatinal strain Dick 2, and non-scarlatinal fibrinolytic strain H-1, were cultured in lots of the same medium. Fractions were then isolated as for the preparations of the NY5 strain. No yellow "oil" was noted in the working up of two lots of Dick 2 filtrate. The chemical analyses of the fractions of the two strains were similar to those of the NY5 strain (see table 2).

Analytical methods

The preparations were analyzed essentially as for the peptone polysaccharide. Because they were hygroscopic, samples were dried *in vacuo* and weighed in micro "pigs." Uronic acid anhydride was determined by the microgravimetric procedure of Burkhart, Baur and Link (1934). The yields of substance were too small to attempt to identify the type of hexosamine or uronic acid.

Kendall, Heidelberger and Dawson (1937) have isolated and analyzed a "serologically inactive polysaccharide" believed to be a bacterial fraction of the mucoid phase of the NY5 and other strains of streptococci. Comparison of their analyses with those of our preparations indicate a basic identity. Kendall *et al.* offer as proof of bacterial origin, their inability to obtain appreciable amounts of polysaccharide from the culture medium or its components (lean beef infusion and Bacto-peptone). Likewise, we have been unable to obtain uronic acid polysaccharide from medium components. However, a polysaccharide in small amounts has been recovered from beef-heart infusion, which gave a positive test for hexosamine after acid hydrolysis and showed a negative uronic acid test.

Because the analysis of the uronic acid polysaccharide was

strikingly similar to that of Jorpes (1935) for "heparin," various preparations were tested for an anti-coagulant action. The results obtained by experiments on human blood indicated that our products had slight anti-coagulant power. In addition, a rapid sedimentation of the red blood cells (irrespective of type) occurred prior to clot formation. Slide preparations examined with the microscope revealed that a form of agglutination had resulted. The anti-coagulant action of the uronic acid polysaccharide from the weakly fibrinolytic strain NY5 and the more strongly fibrinolytic strains Dick 2 and our own strain H1, suggests that it is the true anticoagulant substance of purified fibrinolysin preparations mentioned by Tillett (1938, p. 191) in a recent review.

TABLE 3

Anti-coagulant and agglutinative action of uronic acid polysaccharide preparations

AMOUNT OF FRACTION TESTED	SEDIMENTATION TIME			CLOTTING TIME		
	Strain NY5	Strain Dick 2	Strain H1	Strain NY5	Strain Dick 2	Strain H1
mgm.	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
5.0		2.5	2.5		60	120
2.0	2.5	3	3	18	55	55
1.0		6	3		50	40
0.5		14	14		18	23
0.0	No sedimentation in 6 minutes			6		

Experimental

Varying amounts of polysaccharide preparations from culture filtrates of three strains of organisms were dissolved in 0.5 ml. of saline in 8 x 1 cm. tubes. To each, 0.5 ml. of fresh human blood was added. The time required for complete sedimentation and for coagulation were noted and are recorded in table 3.

III. THE YELLOW OIL

The yellow "oil" mentioned in the previous section was not precipitated from uninoculated broth or its ingredients, or from Dick 2 preparations. However, we have no conclusive proof of its bacterial origin.

One milliliter of "oil" in 9 ml. of water, in which it was readily soluble, rotated the plane of polarized light + 0.05°. The "oil" gave a positive Molisch test, a positive biuret, and on standing in the icebox, a precipitate formed which redissolved at room temperature. These properties have suggested the possibility that the substance may be the yellow "enzyme" of Warburg and Christian (1933) which at one stage is described as an oil when precipitated by acetone. Lack of facilities have prevented its identification.

IV. SYRUPY PROTEIN PRODUCTS FROM MEDIUM

In the isolation of the polysaccharide and associated toxin detailed in the preceding sections, small volumes of alcohol were used as precipitant. If larger volumes of alcohol were added, the remainder of the toxin was precipitated together with brown, syrupy, protein products, which dried to brownish powders. These fractions gave strongly positive biuret reactions. No further analyses were made.

By alcoholic precipitation of a growth-supporting peptone, a medium was prepared from which considerable quantities of protein syrups were removed. With the completed medium, which contained the residue from the alcohol-soluble fraction, protein syrups could be precipitated only by the addition of many volumes of alcohol. Streptococcic culture filtrates were then prepared, and efforts made to isolate toxin free from extraneous substances. Surprising results were obtained. Three to five volumes of alcohol precipitated no erythrogenic toxin (as well as no protein syrups). Toxin was precipitated only when the alcohol was raised to such a concentration that protein syrups began to precipitate.

V. THE "NUCLEOPROTEIN" OF CULTURE FILTRATES

Ando, Kurauchi, and Nishimura (1930) have reported that streptococcus exotoxin fractions isolated from culture filtrates contained a "nucleoprotein" which could be precipitated and separated from the true toxin by acidification to pH 4.0. These workers believed that the acid precipitable fraction was bacterial

in origin and presented evidence to show that it was responsible for false-positive Dick reactions. Such acid-precipitable proteins were encountered in streptococcic culture filtrates described in Parts I and II. By use of similar methods, we have also demonstrated an acid-precipitable protein in uninoculated medium and its components, meat infusion and certain peptones. Consequently, the absence of acid-precipitable proteins from the medium must be assured before assigning a bacterial origin for similar substances from streptococcic filtrates. Inasmuch as precipitation by acid has been used extensively for the preparation of toxins (Eaton, 1938, p. 18), the same precaution applies to filtrates of other organisms.

Experimental

Concentrates of uninoculated medium or meat infusion were precipitated with two volumes of alcohol. On acidification to pH 4.0 of the water soluble portion of this precipitate, a protein was reprecipitated. Parke-Davis peptone and other polysaccharide-containing peptones examined for the polysaccharide gave a heavy precipitate with acid at this stage. It is of interest to note that commercial pepsin and pancreatin fractions also gave copious precipitates with acid by the same method.

DISCUSSION

From the above experiments, it is evident that any investigation on the isolation of bacterial products from routine mediums may be complicated by the presence of some of the enumerated products. For example, we have encountered the peptone polysaccharide in preparing purified fibrinolysin from hemolytic streptococcic filtrates by the alcohol method of Garner and Tillett (1934). It is probable that other investigators have had similar experiences. Goebel (1938) recently isolated the polysaccharide from other brands of peptone, and has described the manner in which it interferes with bacteriological and serological experiments as far as the pneumococcus is concerned.

In our experiments, the erythrogenic toxin of the NY5 strain of scarlatinal streptococcus was precipitated from the usual

culture filtrates only by adsorption on a variety of precipitated fractions. Further separation of the toxin from these fractions seemed impractical. Therefore, these mediums and methods have been abandoned and others developed which are more successful for the isolation of a purer erythrogenic toxin (Stock, 1939).

SUMMARY

A group of extraneous products encountered in preliminary experiments aimed at isolation of erythrogenic toxin from streptococcic culture filtrates is presented. These are, namely, a peptone polysaccharide, a uronic acid polysaccharide, a yellow "oil," syrupy protein products from medium, and an acid-precipitable protein.

The polysaccharide derived from peptone is described, its analysis given, and its possible origin suggested as pepsin or trypsin preparations. In this connection a polysaccharide from pancreas is described. The peptone polysaccharide characterizes those peptones able to sustain streptococcic growth without added meat infusion, but it is not an essential growth factor.

Uronic acid polysaccharides obtained from streptococcic filtrates of several strains of organisms are described, compositions given, and their anticoagulant property and agglutinative action on red blood cells shown. The similarity of these preparations to serologically inactive "mucoid" polysaccharides of Kendall, Heidelberger, and Dawson is pointed out.

A yellow "oil" that was also precipitated from the filtrates is described.

Syrupy protein products were precipitated from culture mediums and from streptococcic filtrates by the addition of several volumes of alcohol. A peptone medium largely free of protein syrups is described.

An acid precipitable protein has been isolated from uninoculated medium. This substance should not be overlooked in evaluating the presence of so-called "nucleoprotein" in bacterial filtrates from streptococci and other organisms.

Erythrogenic toxin was associated with some of these sub-

stances when precipitated from culture filtrates. Toxin was isolated from the filtrates only when these fractions were present to act as adsorbent agents during precipitation by alcohol.

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STUDIES WITH THE AGAR CUP-PLATE METHOD
I. A STANDARDIZED AGAR CUP-PLATE TECHNIQUE

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Official Circular 198 (Ruehle and Brewer, 1931) gives directions for performing the agar cup-plate test. However, in the limited literature dealing with the subject, no mention is made of the influence of varying the volume of agar, the amount of inoculum, the hydrogen ion concentration or other factors. In the course of other studies with the agar cup-plate method (Rose and Miller, 1939; Miller and Rose, 1939) it was learned that a slight modification in procedure caused a considerable difference in the experimental results. It therefore became necessary to study the influence of variations in technique in greater detail. The object was to standardize the method so that more constant data could be obtained. The present communication deals with this study.

TECHNIQUE

A simple device is used to remove the disc of agar from the medium. This instrument[1] consists of a thin-walled stainless steel cylindrical chamber measuring 2.5 cm. in length and having a diameter of 1.5 cm.; the cutting edge is bevelled on the inside. A capillary metal tube, about 10 cm. long, is attached to the bottom of the cylindrical chamber. The chamber is sterilized by dipping in alcohol and flaming. Placing the open end of the chamber on the surface of a poured agar plate, the disc is cut easily with slight pressure. A finger is then placed over the tip

[1] Made by Mr. Charles Simpson, Laboratories of the Philadelphia General Hospital.

of the capillary tube, as in pipetting; the disc of agar is gently pulled slightly to one side and removed. With practice, no cracks are made in the walls of the cup.

The ordinary inoculating loop is not entirely satisfactory for removing small pieces of agar for subculture. A *micro-spoon*[2] is convenient for such purposes. The spoon is formed at the end of a piece of 10 per cent iridium-platinum No. 19 B. & S. gauge wire, 4 cm. long; it measures 2 mm. in diameter and has a shallow concavity approximately 0.75 mm. deep. The handle is mounted on a standard needle holder. About 30 seconds are required to cool the micro-spoon after sterilization in an open flame.

When it is necessary to determine whether the zone of inhibition of bacterial growth is bactericidal or bacteriostatic, a small piece of agar is removed with the micro-spoon, transferred to 10 ml. of broth, and incubated at 37°C. Readings are made at the end of 48 hours. Growth in a subculture tube indicates inhibitory rather than germicidal action of the antiseptic.

The antiseptics used in this study were limited to a single member of each of three groups. Carbolic acid was chosen for the phenol group because it has long been used as a standard. The dilution of 1:20 was employed because this was the weakest dilution which produced a zone free of bacterial growth of approximately 3 mm.; this size was found to be a convenient minimal one for accurate measurement. Mercury bichloride and crystal violet,[3] each in 1:1,000 dilution, were taken as representatives of the heavy metals and antiseptic dyes respectively. In one instance, neutral acriflavine[3] was added, to illustrate further the effect of the hydrogen ion concentration of the medium on the size of the zone. All hydrogen-ion-concentration determinations were made colorimetrically.

Except where experimental conditions required a change in procedure, the following technique was employed. Beef extract agar (Ruehle and Brewer, 1931) was kept melted at 48°C. in a con-

[2] Made by J. Bishop and Company, Platinum Works, Malvern, Penna.

[3] Supplied through the courtesy of National Aniline and Chemical Company, New York, N. Y.

stant temperature incubator. Each 30 ml. amount of 1.5 per cent extract agar at pH 6.8 was inoculated with 0.1 ml. of an 18-hour growth of the organism being studied. The cultures were standardized to a density of 1 billion organisms per milliliter with a barium sulphate nephelometer. Unless otherwise specified, our WP3 strain of *Staphylococcus aureus* was employed. As soon as the bacteria were added, the container was rotated to insure uniform distribution of organisms. Approximately 30 ml. amounts were poured into standard 100 x 15 mm. petri dish bottoms. The dishes were covered with Coors porcelain lids glazed on the outside. The porcelain lids absorbed water of condensation and prevented the deposition of moisture on the test medium. When the agar solidified, one disc of agar was usually cut from each plate. At times it was desirable for comparative purposes to cut three or four agar cups in a single dish. The standard volume of antiseptic employed was 0.2 ml. of a given dilution. The agar cup-plates were incubated in an upright position at 37°C. for 24 hours and readings were then made. All tests were performed in duplicate.

EXPERIMENTAL

Experiment 1. The amount of agar that has been recommended (Ruehle and Brewer, 1931) for the agar cup-plate test is 15 to 20 ml. However, when pouring plates, it is rather difficult to gauge the precise amount delivered. Furthermore, it was soon learned that a deep cup minimized the danger of spilling the antiseptic over the surface of the medium. As no data on the relationship of medium thickness to zone size was available, the question was answered as follows: A known quantity (usually 180 ml.) of 1.5 per cent agar was inoculated with 0.1 ml. of *Staphylococcus aureus* culture for each 30 ml. of medium. Varying quantities of agar from this one batch were pipetted into petri dishes and 0.2 ml. amounts of each antiseptic were placed in the cups. Uniform results were obtained when the agar volume was 25 to 35 ml. (table 1). We chose the 30 ml. amount as this produced a well of satisfactory depth.

Experiment 2. The sensitivity of the agar cup-plate method

TABLE 1

Influence of thickness of medium on zone size

ANTISEPTIC	AGAR	ZONE
	ml.	*mm.*
Phenol (1:20)	15	6
	25	3+
	30	3+
	35	3+
Mercury bichloride (1:1000)	15	11
	25	11
	30	11
	35	11
Crystal violet (1:1000)	15	8
	25	8
	30	8
	35	8

TABLE 2

Variation in the number of organisms

ANTISEPTIC	STAPH. AUREUS	ZONE
	ml.	*mm.*
Phenol (1:20)	0.1	3+
	0.2	3+
	0.3	3+
	0.4	3
	0.5	3
	1.0	2+
Mercury bichloride (1:1000)	0.1	11
	0.2	10+
	0.3	10
	0.4	10
	0.5	9
	1.0	8+
Crystal violet (1:1000)	0.1	8+
	0.2	8
	0.3	7
	0.4	6+
	0.5	6+
	1.0	6

to various numbers of bacteria was determined. An 18-hour growth of *Staphylococcus aureus* was standardized to one billion organisms per milliliter and amounts from 0.1 to 1 ml. were employed in the tests. It is evident that slight variations in the number of organisms (table 2) do not produce striking differences in the zone of inhibition of growth. One-tenth of a milliliter of a suspension of one billion organisms per milliliter usually yielded

TABLE 3

Varying the amount of antiseptic

ANTISEPTIC	VOLUME	ZONE
	ml.	*mm.*
Phenol (1:20)	0.1	2+
	0.2	3+
	0.3	4+
	0.4	6
	0.5	7
Mercury bichloride (1:1000)	0.1	11
	0.2	11+
	0.3	12
	0.4	12
	0.5	12
Crystal violet (1:1000)	0.1	7
	0.2	8
	0.3	8+
	0.4	8
	0.5	8+

a readily discernible line of demarcation between the zone of inhibition and the growth of bacteria. This amount of culture was taken as a standard in all subsequent studies.

Experiment 3. In order to test the influence of the amount of antiseptic on zone size, different amounts of a constant dilution of each antiseptic were placed in agar cups. The use of a graduated 1 ml. pipette gave more uniform results than the advocated "six drops." Although a drop more or less of antiseptic ordinarily does not cause marked changes in the results (table 3) it appears desirable to use a constant volume of a given dilution of anti-

septic in comparative studies. It was found that 0.2 ml. amounts were satisfactory.

Experiment 4. Different agar concentrations may yield varying results with some antiseptics (table 4). This difficulty may be obviated by using 1.5 per cent agar routinely. Our stock agar was usually prepared in a 3 per cent concentration. This was done in order to permit dilution up to 50 per cent with blood or serum. If an amount of protein solution less than 50 per cent were needed, saline or a saline-protein mixture was added to bring the agar dilution to 1.5 per cent. In another experiment with

TABLE 4

Effect of varying agar concentration

ANTISEPTIC	AGAR	ZONE
	per cent	*mm.*
Phenol (1:20)	3.0	3+
	2 0	3+
	1.5	3+
Mercury bichloride (1:1000)	3.0	11
	2 0	11
	1.5	11
Crystal violet (1:1000)	3 0	6+
	2 0	7+
	1 5	8

Staphylococcus aureus, it was found that a 50 per cent dilution of 3 per cent agar with saline gave zones identical in size with those obtained when an original 1.5 per cent agar was used.

Experiment 5. Although a difference in medium does not cause a marked change with phenol and crystal violet, the zone size with mercury bichloride is practically 50 per cent larger in extract agar than in beef infusion agar (table 5). This difference in results is probably attributable to the different amounts of protein in the media.

Experiment 6. To check the results obtained in Experiment 5, horse serum was added to extract agar to yield a 10 per cent serum concentration. Material from the same batch of plain extract

agar was used as a control. The comparative zones are shown in table 6; the effect of added protein is obvious.

Experiment 7. The size of the zones produced by phenol and crystal violet remained unchanged at three pH levels (table 7).

TABLE 5

Comparison of infusion with extract agar

ANTISEPTIC	ZONE	
	Extract agar	Infusion agar
	mm.	*mm.*
Phenol (1:20)	3+	3
Mercury bichloride (1:1000)	11	7
Crystal violet (1:1000)	8	7

TABLE 6

Influence of added serum

ANTISEPTIC	ZONE	
	Plain agar	10% serum agar
	mm.	*mm.*
Phenol (1:20)	3+	2+
Mercury bichloride (1:1000)	11+	6
Crystal violet (1:1000)	8	5+

TABLE 7

Hydrogen ion concentration of the medium

ANTISEPTIC	ZONE		
	pH 6.0	pH 7.0	pH 8.0
	mm.	*mm.*	*mm.*
Phenol (1:20)	3+	3+	3+
Mercury bichloride (1:1000)	14	11	10
Crystal violet (1:1000).	9	9	9
Acriflavine (1:1000)	4	5	6

On the other hand, with increasing alkalinity, the mercury compound showed a decrease in zone size while the dye, neutral acriflavine, revealed a definite increase in potency. This experiment emphasizes the importance of controlling and designating the

hydrogen-ion concentration of the medium in the agar cup-plate method.

Experiment 8. A virulent culture of *Staphylococcus aureus* (strain WP3) was selected for our tests. This organism grows homogeneously in broth and has a more golden color than the official F.D.A. strain but conforms with the F.D.A. requirements for the phenol coefficient test. The two strains gave the same size zone with the three antiseptics employed, (table 8) while

TABLE 8

Influence of bacterial strain

ANTISEPTIC	STAPH. AUREUS	ZONE
		mm.
Phenol (1:20)	F.D.A.	3+
	WP3	3+
	Smith	4
	HB-40	4
	W	4+
Mercury bichloride (1:1000)	F.D.A.	11
	WP3	11
	Smith	10
	HB-40	12
	W	12+
Crystal violet (1:1000)	F.D.A.	8
	WP3	8
	Smith	7+
	HB-40	7+
	W	8

other strains tested failed to give uniformly comparable zones of inhibition of growth. Obviously, it is important to use the official strain of *Staphylococcus aureus* or one that has been shown by actual test to have the same phenol resistance.

Experiment 9. Eight different bacteria, studied under identical experimental conditions, yielded a wide variety of zone size (table 9). It is clear that the test organism must be designated when comparative studies are made with the agar cup-plate method.

Experiment 10. The official phenol coefficient test requires the daily transfer of a standard loopful of culture into 10 ml. of broth for five consecutive days before doing the test. The question arose whether a similar technique was necessary for the agar cup-plate method. Accordingly, the WP3 *Staphylococcus aureus* was subcultured daily for five days in broth. The first subculture

TABLE 9

Data obtained with various organisms

ORGANISM	ZONE		
	Phenol (1·20)	Mercury bichloride (1:1000)	Crystal violet (1:1000)
	mm.	*mm.*	*mm.*
Staph. albus	1+	6+	7
Staph. aureus (WP3)	3	7	7
Strep. hemolyticus	7	7+	5
Pneumococcus (Type 1)	9	7	7
B. diphtheriae	8	4+	8
B. subtilis	3+	8	6+
B. typhosus	3	4	1+
B. coli	3+	3+	1

Beef infusion agar, pH 7.6, was used for all media in this experiment.

TABLE 10

Daily transfers versus 18-hour growths

ANTISEPTIC	ZONE	
	5-day transfer	18-hour growth
	mm.	*mm.*
Phenol (1:20)	3+	3+
Mercury bichloride (1:1000)	11+	11+
Crystal violet (1:1000)	8+	8+

was from our stock culture kept on an extract agar slant at room temperature and subcultured to agar at least once a month. The same organism was transferred from the stock culture to 25 ml. of broth and grown for 18 hours. The results of the tests with the two cultures are shown in table 10. The 18-hour growth, adjusted nephelometrically before using, yielded zones similar

in size to those produced by a *Staphylococcus aureus* culture which had been subcultured daily for five days.

Experiment 11. A parallel series of agar cup-plates was studied at three temperature levels: 37°C., 34°C., and 24.5°C. A variation in temperature causes a difference in the results obtained (table 11). A temperature of 37°C. was selected as a convenient standard for our tests.

Experiment 12. Under ordinary working conditions, bacteria are added to the agar, plates are poured, agar cooled, cups cut, an antiseptic added at once, and the preparation incubated at

TABLE 11

Influence of temperature of incubation

ANTISEPTIC	TEMPERATURE	ZONE
	°C.	*mm.*
Phenol (1:20) ..	37	3
	34	3+
	24.5	4+
Mercury bichloride (1:1000)	37	11
	34	11
	24.5	15
Crystal violet (1:1000)	37	8
	34	8+
	24 5	9

37°C. However, when many antiseptics are being tested simultaneously, a moderate interval of time may elapse between the pouring of the seeded agar and the addition of the antiseptic. Although this experiment shows that a lapse of five hours at room (24.5°C.) or refrigerator (14.5°C.) temperature has no obvious influence on the results (table 12), it is probably desirable to have the test plates ready for incubation within an hour after seeding.

On the basis of the data obtained in this study with three types of antiseptics, the following technique is suggested for the agar cup-plate method:

1. Use a "standard" organism. If *Staphylococcus aureus* is the test organism, use either the F.D.A. strain or one known to possess the same resistance to phenol.

2. Grow the bacteria for 18 to 24 hours in broth and standardize the suspension to one billion organisms per milliliter.

3. Extract agar, pH 6.8, may be employed. Some bacteria require the use of beef infusion agar, pH 7.4. Both types of medium are satisfactory, but the medium as well as the pH must be designated.

4. The final agar concentration should be 1.5 per cent. A 3 per cent agar, diluted to 1.5 per cent agar with saline, is satisfactory. Such an agar can also be reduced in concentration with protein (serum, blood) or protein-saline mixtures. In this manner protein-agar mixtures containing up to 50 per cent protein solution may be conveniently made for special studies.

TABLE 12

Time and temperature factors before addition of antiseptic

ANTISEPTIC	LAPSE OF TIME BEFORE ADDING ANTISEPTIC		
	0 hour	5 hours, 24.5°C.	5 hours, 14.5°C.
Phenol (1:20)	3+	3+	3+
Mercury bichloride (1:1000)	11+	12	11
Crystal violet (1:1000)	8	8	8

5. Seed the agar medium on the basis of 0.1 ml. of the standard suspension of organisms for each 30 ml. volume of medium. The temperature of the agar should be about 48°C., that is, the temperature ordinarily used in making blood agar medium.

6. Pour approximately 30 ml. of seeded agar medium into a standard 100 x 15 mm. petri dish bottom. Cover the petri dish with a Coors porcelain lid glazed on the outside.

7. After the agar solidifies, remove a disc of agar measuring 1.5 cm. in diameter. In some instances it is feasible and desirable to prepare three or more cups from a single plate.

8. Prepare appropriate dilutions of the antiseptic. Using a graduated 1 ml. pipette, place 0.2 ml. of a given dilution in each cup.

9. The antiseptic should be added and the plates should be ready for incubation within one hour from the time the bacteria are added to the agar medium.

10. Incubate test plates upright at 37°C. for 24 hours and then measure the width of the zone of inhibition of bacterial growth with a transparent ruler. The use of a hand lens in measuring the zones is an aid in obtaining accurate results.

Experiment 13. The technical procedure outlined above was checked as follows: Fresh solutions of the three antiseptics used

TABLE 13

Consistency of results obtained with the agar cup-plate method

DATE	ANTISEPTIC	ZONE	
		Plain agar	10% horse serum agar
		mm.	mm.
6/11	Phenol (1:20)	3	2+
6/12		3+	2
6/15		3+	2+
6/16		3+	2+
6/17		3+	2+
6/18		3+	2+
6/19		3+	2
6/11	Mercury bichloride (1:1000)	10+	5+
6/12		11+	6
6/15		10+	6
6/16		11+	6
6/17		11+	6
6/18		11	6
6/19		11	5+
6/11	Crystal violet (1:1000)	8	5+
6/12		7+	6
6/15		7+	5+
6/16		8	6
6/17		8	5+
6/18		8	5+
6/19		8	5+

in this study were prepared. The WP3 strain of *Staphylococcus aureus* was employed. Tests were then performed in duplicate using plain extract agar and extract agar containing 10 per cent horse serum. The same technique was followed on six other days and the readings obtained each day were recorded in table 13. An examination of this table shows that comparatively consistent

results can be obtained when all the technical requirements of the test are observed.

Other studies pertaining to the agar cup-plate method will be reported in separate communications (Rose and Miller, 1939; Miller and Rose, 1939).

CONCLUSIONS

A study was made of the agar cup-plate method of testing antiseptics. It was found that various factors may influence the results. Consequently, a standardized technique was developed. This technique is comparatively simple and yields consistent results with three representative antiseptics: phenol, mercury bichloride, and crystal violet.

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STUDIES WITH THE AGAR CUP-PLATE METHOD

III. The Influence of Agar on Mercury Antiseptics

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Using a standardized agar cup-plate technique (Rose and Miller 1939a), it was found that mercury antiseptics showed a progressive loss of activity as the blood content of the agar medium was increased (Rose and Miller, 1939b). The question arose whether the agar had inactivated a given amount of the antiseptic; this phenomenon could have been masked by the more pronounced activity of the blood. In order to answer this question, experiments were performed in which extract agar, blood, bacteria, and antiseptic were mixed in definite proportions. Duplicate experiments were then made in which extract broth was substituted for the agar medium. It was clear that if the blood-broth and blood-agar mixtures showed the same antibacterial properties for a given amount of antiseptic, the loss of antiseptic potency could not be attributed to the mere presence of agar in the medium, but would be related solely to the variable blood concentration.

TECHNIQUE

A 3 per cent beef-extract agar, pH 6.8, was used. After dilution with blood, blood-saline mixtures, and antiseptic solutions, the final agar concentration was 1.5 per cent. Defibrinated horse blood[1] was added so that final concentrations of 1, 5, 10, 25, and 50 per cent blood were obtained. An 18-hour broth culture of *Staphylococcus aureus* (WP3 strain) was seeded in the

[1] Supplied through the courtesy of Sharp and Dohme, Glenolden.

blood-antiseptic mixtures in the proportion of 0.1 ml. for each 30 ml. of medium. Aqueous solutions of merthiolate,[2] metaphen, mercurochrome, and mercury bichloride were prepared to yield final concentrations varying from 1:1,000 to 1:1,000,000, when diluted by the medium. Then, 0.5 ml. amounts of the antiseptic dilutions were distributed into a series of test tubes. Two milliliters of the seeded blood-agar mixtures were added to the prepared 0.5 ml. amounts of antiseptic. The tubes were rotated vigorously to insure uniform dispersion of the ingredients before solidification occurred. After incubation for 24 hours, small pieces of the various seeded antiseptic mixtures were removed with a platinum microspoon (Rose and Miller, 1939a) and transferred to 10 ml. amounts of broth (Ruehle and Brewer, 1931). Subcultures into a second tube of broth were made when the antiseptic concentration appeared to be high enough to exert bacteriostatic effects. The subculture tubes were incubated at 37°C. for 48 hours and then examined for growth.

The procedure outlined above was repeated with the exception that beef-extract broth, pH 6.8 (same formula as used for subculture tubes), was substituted for the agar medium. The blood cells in these mixtures settled to the bottom of the tubes during incubation. However, the tubes were shaken thoroughly before subcultures were made to determine the bactericidal level. A standard 4 mm. platinum loop was used for subculturing. The highest dilution of antiseptic which killed *Staphylococcus aureus* (indicated by no growth in the subculture tube) in each blood concentration was taken as the end point.

Graphs were made by plotting logarithmically the weakest antiseptic dilution which killed (ordinate) against the corresponding blood concentration (abscissa). Metaphen and mercury bichloride yielded practically identical results in the blood-broth and blood-agar tests (figures 1 and 2). The data for merthiolate and mercurochrome are shown in figures 3 and 4.

The results of these experiments show that agar exerts no obvious inhibiting effect on mercurial antiseptics. Furthermore,

[2] Supplied through the courtesy of Eli Lilly Co., Indianapolis, Indiana.

the graphs reveal the limits of activity of the various antiseptic dilutions in relation to the blood concentration. For example,

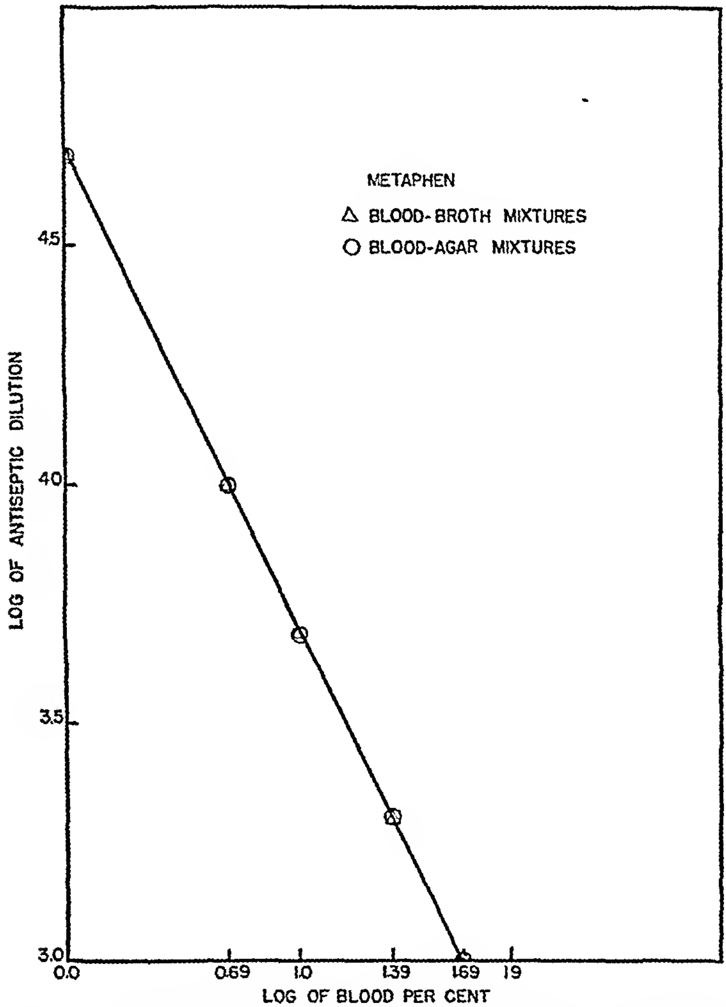
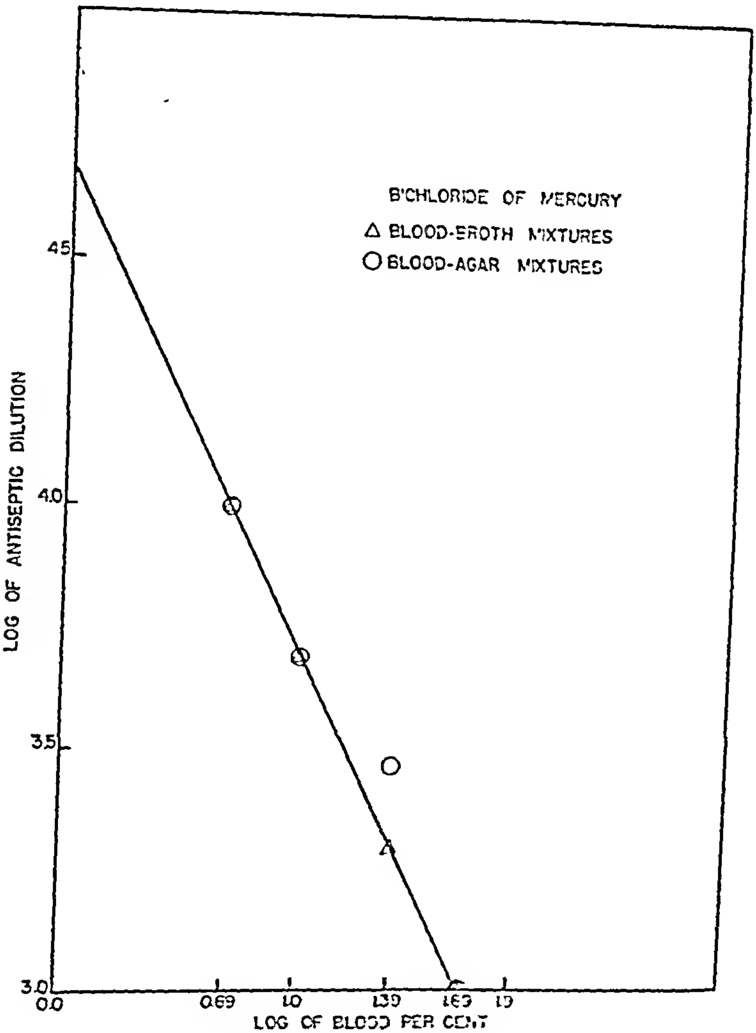


FIG. 1. DILUTIONS OF METAPHEN KILLING STAPHYLOCOCCUS AUREUS IN BLOOD-BROTH AND BLOOD-AGAR MIXTURES IN THE PRESENCE OF VARYING PERCENTAGES OF BLOOD

metaphen is bactericidal in a 1 to 10,000 dilution when the blood concentration is 5 per cent or less. At the same antiseptic dilu-

tion, growth occurred whenever the blood content of the medium was above the 5 per cent level. These observations indicate that



Figs. 2, 3 and 4 show comparable data obtained with three other mercurials

as the amount of blood increases, the quantity of mercury antiseptic needed to kill *Staphylococcus aureus* becomes greater. It follows that a statement concerning the antiseptic potency of a

given mercurial may be meaningless unless the *blood content* of the test medium is designated. Such a conclusion is, in general, in

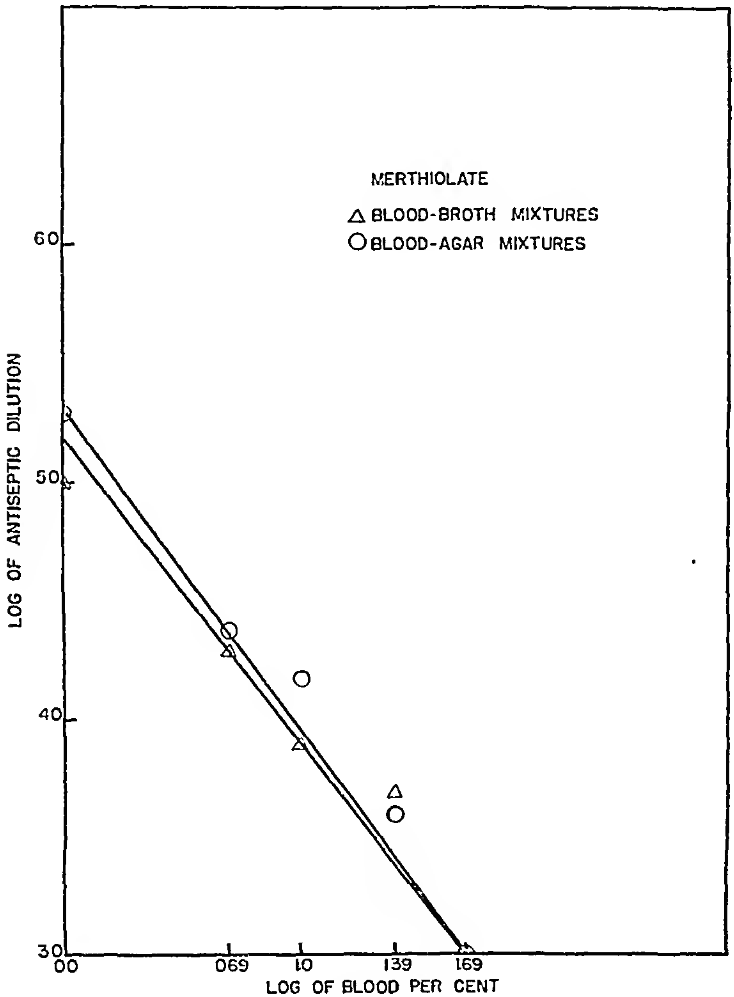


FIG. 3

harmony with other reports (Simmons, 1928; Birkhaug, 1930; Smith, Czarnetzky and Mudd, 1936; Rose and Miller, 1939b and others).

The experimental data[3] shown in figures 1 to 4 are satisfied by the formula $y = Kx^n$ (Power Law) for blood values from 1

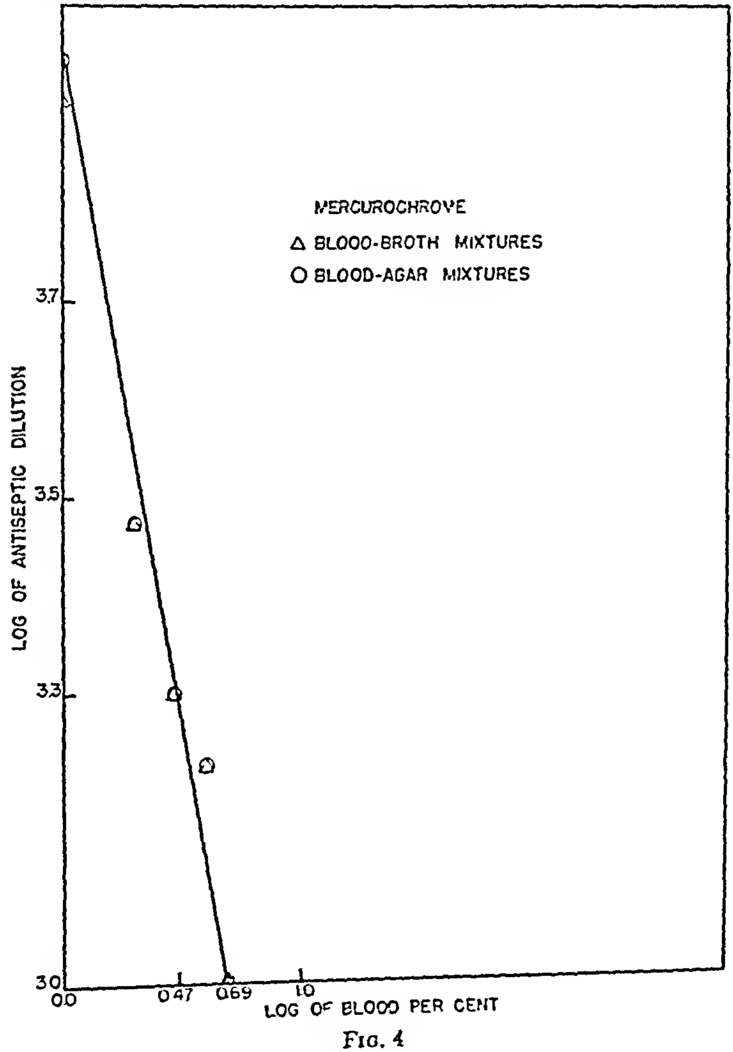


FIG. 4

to 50 per cent. (y, antiseptic dilution; x, blood concentration at which dilution y is bactericidal; K and n, constants for each antiseptic).

[3] We are indebted to Mr. Charles Robb for his invaluable assistance in the mathematical treatment of the data.

The usefulness of the formula may be illustrated in the case of mercury bichloride as follows: The value of n when calculated at $x = 25$, $y = 2{,}000$ and $x = 5$, $y = 10{,}000$ is -1. Hence,

$$y = Kx^{-1} = \frac{K}{x} \tag{1}$$

Solving for K when $x = 5$ and $y = 10{,}000$, yields $K = 50{,}000$. Hence,

$$y = \frac{50{,}000}{x} \tag{2}$$

Let us assume that we wish to determine the maximum per cent blood that can be used effectively with a 1:20,000 dilution of mercury bichloride. Substituting for y in (2)

$$20{,}000 = \frac{50{,}000}{x}$$

therefore $x = 2.5$ per cent blood.

Such a solution would be in accord with our experimental data as shown in figure 2.

The method used to compare the relative efficiency of the mercurials on the basis of their mercury content may be illustrated as follows: mercury bichloride was effective against *Staphylococcus aureus* in a 1:1,000 dilution up to the 50 per cent blood level. Inasmuch as this compound contained 73.8 per cent mercury, 2.5 ml. of the 1:1,000 dilution contained 1.8 mgm. of mercury. In a similar way, the mercury content[4] of the four antiseptics was calculated for each dilution which was bactericidal in the presence of the maximum amount of blood. The results were then plotted logarithmically as shown in figure 5.

It is interesting to compare the four mercurials at a given mercury level. For example, when 1 mgm. of mercury was present, merthiolate was active when the medium contained up to 63 per cent blood, metaphen up to 35 per cent blood, mercury

[4] Calculations were based on the following data: Merthiolate 48.8 per cent Hg; mercury bichloride 73.8 per cent Hg; metaphen 57 per cent Hg; and mercurochrome 26 per cent Hg.

bichloride up to 27 per cent blood and mercurochrome up to 8 per cent blood. Although all of the mercurials are inactivated by blood according to the same general pattern, it is obvious that the antibacterial properties of these substances are not related directly to the amount of mercury in the compound.

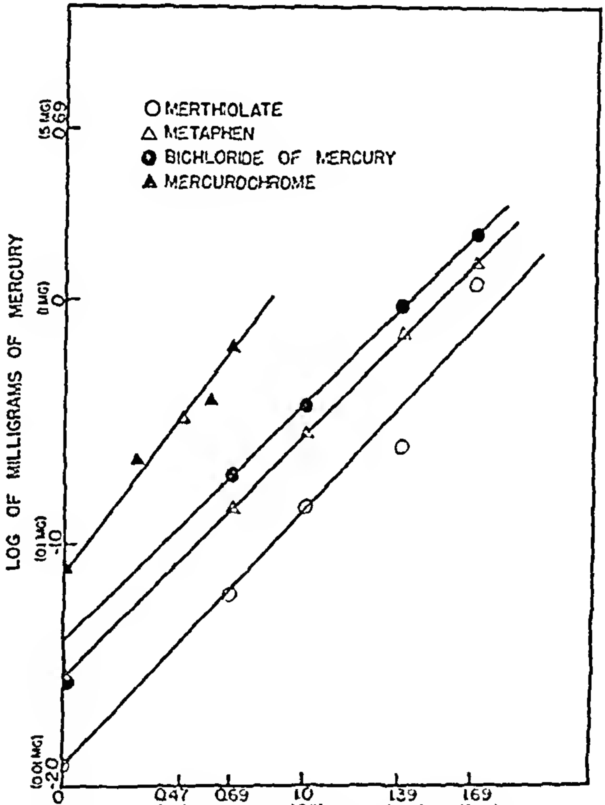


FIG. 5. MILLIGRAMS OF MERCURY REQUIRED TO KILL STAPHYLOCOCCUS AUREUS IN VARYING PERCENTAGES OF BLOOD (CALCULATED FROM BLOOD-BROTH DATA)

SUMMARY AND CONCLUSIONS

Metaphen, merthiolate, mercury bichloride, and mercurochrome were tested for antiseptic activity at various dilutions in broth and agar in the presence of varying amounts of blood.

The test organism was *Staphylococcus aureus* (WP3 strain). Under the conditions of our experiments, the following conclusions appear to be warranted:

1. Agar exerts little or no effect on the antiseptic properties of mercury compounds.
2. The effectiveness of the mercury compounds varies with the dilution of the antiseptic and the blood content of the medium according to the general formula, $y = Kx^n$.
3. At certain critical blood and antiseptic levels the compounds studied have no antiseptic properties.
4. The antiseptic potency is not directly related to the mercury content of the compound.

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THE RELATIONSHIP BETWEEN TEMPERATURE AND THE STREPTOCOCCIDAL ACTIVITY OF SULFANILAMIDE AND SULFAPYRIDINE IN VITRO[1]

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In a recent study of the effect of sulfanilamide upon beta-hemolytic streptococci in peptone broth (White and Parker, 1938), it was reported that bactericidal action could be demonstrated, with 20 mgm. per cent drug concentration, when incubation was carried out at 39°C. or higher. Attempts to obtain a similar bactericidal effect with incubation at 37°C. were unsuccessful. This work was preliminary in nature, lacked highly accurate temperature control, and, for the most part, consisted of tests with a single inoculum of bacteria against a single drug concentration.

In view of the possibility that temperature might be a determining factor in the action of sulfonamide-type drugs, it became desirable to carry out more extensive *in vitro* tests under conditions of precise temperature control.

The present report deals with data bearing on quantitative relationships between drug and bacteria, in terms of bactericidal action, at various temperatures between 30° and 39°C.

METHODS

A uniform test procedure, devised for measuring the antibacterial activity of water-soluble compounds *in vitro*, was used throughout the present study.

[1] This investigation has been aided by a grant from The John and Mary R. Markle Foundation.

Culture

Strain C 203[2] was selected as our standard test culture. This strain is a member of the serological group A (Lancefield), type 1 (Griffith). Daily passage through our test medium at 37°C. was maintained for several months. Test cultures, when required, were prepared by transfer of 1.0 ml. of an 18-hour broth culture into 9.0 ml. of sterile broth with incubation for 6 to 8 hours at 37°C. After several passages in plain broth the culture possessed little or no mouse virulence: mice survived 0.5 ml. of a 1:1000 dilution of the 136th broth passage administered intraperitoneally. The culture from which plain broth passage originated had the high degree of mouse virulence characteristic of this strain.

Medium

Peptone-glucose broth (PD), described in our previous report, has been our standard test medium. This medium is essentially a 2.1 per cent solution of peptones plus 0.1 per cent glucose buffered at a pH of approximately 7.6. Plain PD was used for making test mixtures. Blood broth, made by adding a drop of defibrinated rabbit blood to 9 ml. quantities of plain broth, was used for subculturing test mixtures.

Drug concentrations

Sulfanilamide was weighed into plain broth, dissolved by heating to 100°C., and diluted with similar broth so that the final concentrations of drug, in each test series, consisted of multiples by 0.1 of each member of the arithmetic progression 1000, 800, 600, 400, 200 mgm. per cent down to 0.1, 0.08, 0.06, 0.04, 0.02 mgm. per cent. Thus, a full test series was made up of drug dilutions ranging from 1:100 to 1:5,000,000. 5.0 ml. amounts of each drug dilution were placed in tubes (150 x 16 mm.) which were then plugged with cotton and sterilized by autoclaving at 110°C. for 10 minutes. Drug solutions were allowed to stand over night before inoculation with test culture. Test solutions of sulfapyridine were made in a similar manner,

[2] We wish to thank Dr. Perrin H. Long for his courtesy in supplying this strain.

beginning with a concentration of 100 mgm. per cent. Autoclaving apparently had no effect upon the activity of the drugs.

Test mixtures

Our test culture was diluted with broth out to 10^{-6}, in multiples of 10, to provide six different sizes of test inoculum. For each temperature, six series of the tubes containing decreasing concentrations of drug were inoculated, one series for each culture dilution. The volume of inoculum, in each case was 0.2 ml. which brought the total volume of the test mixtures up to 5.2 ml. The range covered by the drug concentrations in each series was such that both inhibitory and bactericidal end-points were obtained, providing that such points fell within the limits mentioned above. Plain broth tubes, seeded with each different size of inoculum, served as controls and were incubated at each temperature. The initial concentration of bacteria obtained with each size of inoculum was determined by averaging several blood-agar pour plate counts.

Incubation of test mixtures

Incubation of all drug-bacteria mixtures was carried out for 48 hours in water baths[3] with thermoregulator control to ± 0.005 of a degree Centigrade (Bratton, 1939).

Inhibitory and bactericidal end-points

Inhibition, in each test series, was recorded at the 24th and 48th hour of incubation. Turbid growth, in the control tubes and in the tubes at the lower end of the range of drug concentrations, coupled with no growth in the tubes at the upper end of the range, provided a sharp inhibitory end-point which was expressed as the smallest concentration of drug preventing growth. The bactericidal end-point, at 48 hours, was determined by subculturing 1.0 ml. from test mixture tubes, on both sides of the 48-hour inhibitory end-point, into 9.0 ml. of blood broth with incubation at 36°C. for 72 hours. One or two tubes in a series

[3] We are greatly indebted to Dr. A. C. Bratton of this department for the design and construction of these baths.

usually showed inhibition at 48 hours yet yielded positive subcultures. Thus, the bactericidal end-point for each series was generally somewhat higher than the inhibitory end-point. Antibacterial activity, on the part of each compound, was expressed in terms of the minimal bactericidal concentration in milligrams per cent. We have continued to use the term *bactericidal action* in its strict sense of sterilization, according to criteria previously described (White and Parker, 1938).

RESULTS

The procedure described above has been used in this laboratory, during the past year, in several hundred measurements of antibacterial activity. Each test consisted of titration of a compound against a single size of bacterial inoculum at a single incubation temperature. Groups of 12 tests were usually run simultaneously, 6 at each of two different temperatures. The six sizes of inoculum, tested at each temperature, were obtained by diluting the test culture out to 10^{-6}. After inoculating a series of drug concentrations and 5 plain broth control tubes with 0.2 ml. of each culture dilution, suitable dilutions of 1.0 ml. from 3 control tubes were plated, in duplicate, to obtain an estimate of the initial bacterial concentration in the test mixtures. Plate counts, made in this manner, for each group of tests, showed that the average initial concentration of strain C 203 was approximately 50 per ml. in test mixtures which had been inoculated from the 10^{-6} culture dilution.

SULFANILAMIDE

Titration of activity

Sulfanilamide was titrated to determine its bactericidal activity against each of 6 different sizes of inoculum at 9 different incubation temperatures between 30° and 39.25°C. The total number of titrations made with this drug was 223: 6 at 30°C.; 6 at 33°C.; 6 at 34°C.; 21 at 35°C.; 12 at 36°C.; 51 at 37°C.; 49 at 38°C.; 57 at 39°C.; 15 at 39.25°C.

Results of measuring the activity of sulfanilamide, with 54 combinations of inoculum size and temperature, are listed in

table 1. 18 of the 54 titrations, including those carried out at 30, 33 and 34°C., in which little or no bactericidal activity was demonstrable, were established by a single test. Bactericidal end-points for the remaining 36 combinations of temperature and inoculum size have been based upon more than one test, ranging from 2 to 10 repetitions. For these 36 combinations, where two or more titrations were made, the average bactericidal end-point is given, with only the first digit considered significant, because of the wide intervals between adjacent drug concentrations in any test series.

TABLE 1

The bactericidal activity of sulfanilamide against strain C 203 at incubation temperatures between 30 and 39.25 degrees Centigrade

INITIAL BACTERIAL CONCENTRATION	MINIMAL BACTERICIDAL CONCENTRATION OF SULFANILAMIDE IN MGM. PER CENT								
	30 C.	33 C.	34 C.	35 C.	36 C.	37 C.	38 C.	39 C.	39.25 C.
5,000,000	>1000 (1)	>1000 (1)	>1000 (1)	>1000 (3)	>1000 (2)	900 (8)	700 (9)	400 (9)	200 (3)
500,000	>1000 (1)	>1000 (1)	>1000 (1)	>1000 (3)	>1000 (2)	700 (8)	400 (9)	100 (9)	5 (3)
50,000	>1000 (1)	>1000 (1)	>1000 (1)	900 (3)	700 (2)	500 (8)	300 (8)	6 (9)	0.8 (3)
5,000	>1000 (1)	>1000 (1)	>1000 (1)	700 (4)	500 (2)	300 (9)	100 (7)	3 (10)	0.6 (2)
500	>1000 (1)	800 (1)	800 (1)	600 (4)	500 (2)	200 (9)	30 (8)	2 (10)	0.08 (2)
50	1000 (1)	600 (1)	600 (1)	600 (4)	300 (2)	100 (9)	8 (8)	0.8 (10)	0.04 (2)

Figures in parentheses indicate the number of determinations upon which each bactericidal end-point of sulfanilamide is based. Where two or more determinations are indicated, the minimal bactericidal concentration of sulfanilamide is expressed as the average, with only the first digit considered to be significant. Initial bacterial concentrations listed above indicate the average number of bacterial units per ml. of test mixture, as determined by plate counts.

Variations in bactericidal end-points

In table 2 are listed 57 individual test results for the six combinations of inoculum with 39°C. incubation. These results show the variation which one may expect with measurements of this nature. Considered as a whole, the variation in results, for any combination of temperature and inoculum, was such that one could expect the bactericidal end-point to fall within four consecutive drug concentrations. For example, in table 1 the average end-point for an initial bacterial concentration of 5000 per ml. at 39°C. is given as 3 mgm. per cent. From table 2, it may be seen that the variations in the results, upon which this average was based, lie within the limits of the four consecutive

drug concentrations: 4, 2, 1, and 0.8 mgm. per cent. In only 7 of the temperature-inoculum combinations was the variation for repeated tests so great as to fall within four consecutive tubes. Results of repeated tests for all other combinations varied within 3 consecutive tubes, or less.

TABLE 2

Individual test results: The bactericidal activity of sulfanilamide against strain C 203 at 39°C.

NUMBER OF TEST	INITIAL BACTERIAL CONCENTRATION PER ML. OF TEST MIXTURE					
	5,000,000	500,000	50,000	5,000	500	50
1	400	80	6	4	2	1
2	400	200	8	4	2	1
3	400	60	6	4	0.8	0.4
4	400	200	10	0.8	0.8	0.8
5	400	200	10	0.8	0.6	0.4
6				2	2	0.8
7				2	2	1
8	400	100	6	4	2	1
9	400	80	4	4	1	0.8
10	200	60	4	2	2	1
11	400	60	4			

Figures in table above indicate minimal bactericidal concentrations of sulfanilamide in milligrams per cent.

Bactericidal activity

The relationship between bactericidal activity and the temperature at which such activity was determined is shown in chart 1. In this chart the average minimal bactericidal concentration of sulfanilamide, for each size of inoculum, has been plotted logarithmically against temperature. It is evident, from this chart, that (1) the drug was totally inactive at 30°C.; (2) slight activity, against only the smallest bacterial inoculums, was obtained at 33°C.; (3) only slight activity continued to be manifest with increasing incubation temperatures up to 37°C.; (4) a temperature increase of two degrees, from 37 to 39°C., was accompanied by a large increase (about 100-fold) in drug activity against all but the largest bacterial inoculums; and (5)

concentrations of sulfanilamide of 10 mgm. per cent or less were active only at temperatures above 37°C.

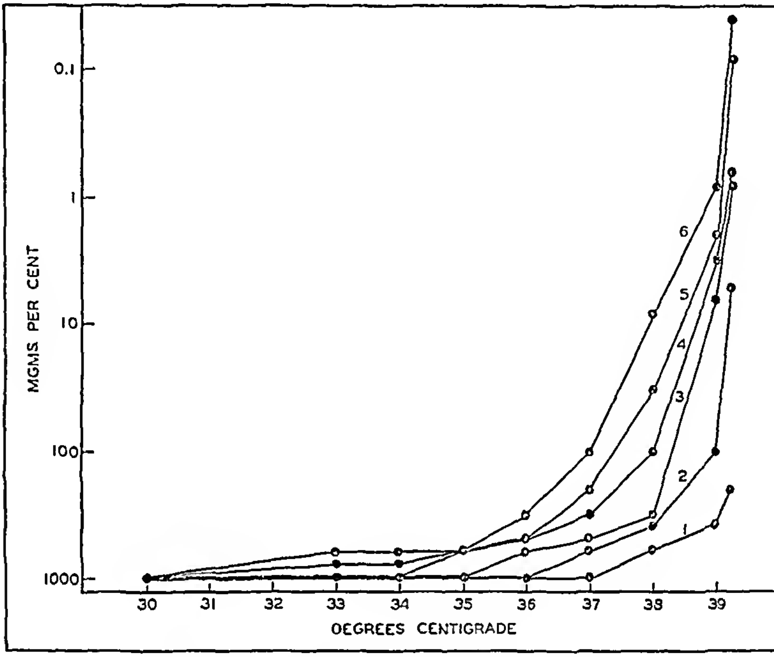

CHART 1. THE RELATIONSHIP BETWEEN TEMPERATURE AND THE BACTERICIDAL ACTIVITY OF SULFANILAMIDE IN PD BROTH

Minimal bactericidal concentrations of drug in milligrams per cent plotted logarithmically against temperatures at which activity was measured. Each curve represents a different size of bacterial inoculum ranging, by multiples of 10, from an average of 50 streptococcus units per milliliter in curve number 6 to an average of 5,000,000 per milliliter in curve number 1.

SULFAPYRIDINE

Titration of activity

Sulfapyridine was titrated against the 6 dilutions of test culture at 6 different incubation temperatures between 30° and 39°C. A total of 89 tests were made with this drug: 6 at 30°C.; 6 at 33°C.; 6 at 36°C.; 24 at 37°C.; 23 at 38°C.; 24 at 39°C.

Results of titrations for activity, in the 36 combinations of temperature and inoculum, are given in table 3. Wherever more

than one determination was made, the bactericidal end-point is expressed as the average, with only the first digit recorded as significant.

TABLE 3

The bactericidal activity of sulfapyridine against strain C 203 at incubation temperatures between 30 and 39 degrees Centigrade

INITIAL BACTERIAL CONCENTRATION	MINIMAL BACTERICIDAL CONCENTRATION OF SULFAPYRIDINE IN MGM. PER CENT					
	30 C.	33 C.	36 C.	37 C.	38 C.	39 C.
5,000,000	>100 (1)	>100 (1)	>100 (1)	>100 (4)	>100 (4)	>100 (4)
500,000	>100 (1)	>100 (1)	>100 (1)	>100 (4)	>100 (4)	20 (4)
50,000	>100 (1)	>100 (1)	>100 (1)	>100 (4)	40 (4)	2 (4)
5,000	>100 (1)	>100 (1)	>100 (1)	>100 (4)	20 (4)	0.8 (4)
500	>100 (1)	>100 (1)	>100 (1)	50 (4)	10 (4)	0.4 (4)
50	>100 (1)	>100 (1)	>100 (1)	20 (4)	5 (3)	0.1 (4)

Figures in parentheses indicate the number of determinations upon which each bactericidal end-point is based. Where two or more determinations are indicated, the minimal bactericidal concentration of sulfapyridine is expressed as the average, with *only the first digit considered to be significant. Initial* bacterial concentrations listed above indicate the average number of bacterial units per ml. of test mixture, as determined by plate counts.

TABLE 4

Individual test results: the bactericidal activity of sulfapyridine against strain C 203 at 39°C.

NUMBER OF TEST	INITIAL BACTERIAL CONCENTRATION PER ML. OF TEST MIXTURE					
	5,000,000	500,000	50,000	5,000	500	50
1	>100	20	2	0.8	0.6	0.1
2	>100	20	2	0.6	0.6	0.2
3	>100	20	2	0.8	0.2	0.08
4	>100	20	2	0.8	0.2	0.1

Figures above indicate minimal bactericidal concentrations of sulfapyridine in milligrams per cent.

Variations in bactericidal end-points

In table 4 are listed 24 individual test results of titrating sulfapyridine against the six different sizes of inoculum at 39°C. From this table, it may be seen that the variation in results is relatively slight, when one considers the limitations inherent in any biological titration.

Bactericidal activity

The relationship between temperature and the bactericidal activity of sulfapyridine is shown in chart 2 in which bactericidal end-points, for each size of inoculum, are plotted logarithmically against incubation temperature. As with sulfanilamide, it is evident from this chart that: (1) the highest concentration of

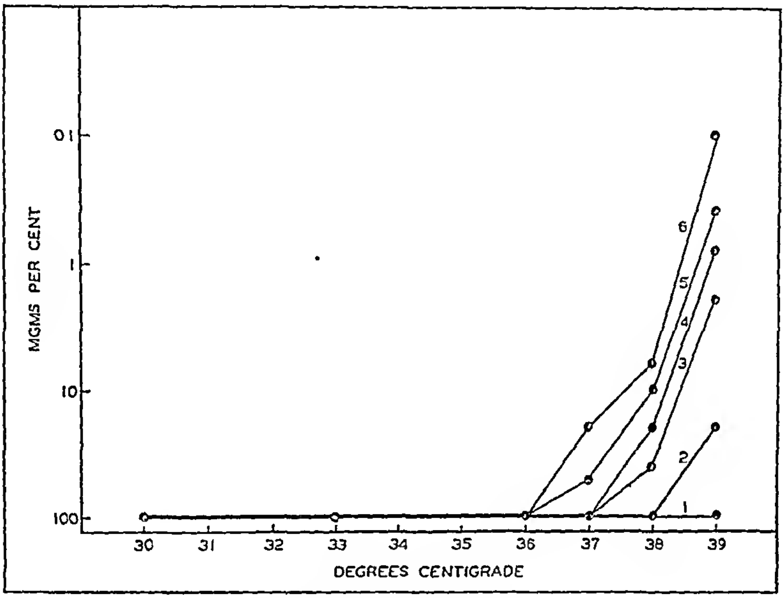

CHART 2. THE RELATIONSHIP BETWEEN TEMPERATURE AND THE BACTERICIDAL ACTIVITY OF SULFAPYRIDINE IN PD BROTH

Minimal bactericidal concentrations of drug in milligrams per cent plotted logarithmically against temperatures at which activity was measured. Each curve represents a different size of bacterial inoculum ranging, by multiples of 10, from an average of 50 streptococcus units per milliliter in curve number 6 to an average of 5,000,000 per milliliter in curve number 1.

sulfapyridine which could be tested was totally inactive at 30°C.; (2) slight activity, against the smaller inoculums, was obtained at 37°C.; (3) an increase in temperature of two degrees, from 37 to 39°C., was accompanied by a large increase in drug activity against all but the largest bacterial inoculum.

COMMENT

The experiments reported here indicate that temperature is a critical accessory factor in the antibacterial action of sulfona-

mide-type drugs *in vitro*. The striking increase in bactericidal power, of both sulfanilamide and sulfapyridine, which accompanies a rise in temperature from 37° to 39°C. is difficult to explain. The possibility that the drug, itself, is directly affected by increasing temperature would appear to be remote since no significant change in activity occurred between 30 and 37°C. Likewise, the view that a direct effect of temperature upon the bacteria renders them more susceptible to bactericidal action does not adequately explain the phenomenon. We have, thus far, been unable to show that streptococci, grown at 39°C., are injured or changed in any way to account for their increased susceptibility to the drugs. It is true that the rate of bacterial growth appears to be optimal at about 37°C. and that a temperature increase to 39°C. decelerates this rate. However, deceleration of growth rate does not, in itself, suffice to explain increased drug activity because at temperatures below 37°C. there occurs a similar deceleration of bacterial growth rate which is accompanied, not by an increase, but by a slight decrease in drug activity. In view of the critical nature of the relationship between temperature and the bactericidal activity of these drugs, it appears to us that its explanation may be found in a study of the effect of temperature upon the metabolic processes of bacteria.

Experimental results, reported by others, which show a quantitative relationship between drug and bacteria are confirmed by our data. However, this relationship appears to be definable in general terms only, and to the extent that, roughly, larger amounts of drug are required to sterilize, as the size of the bacterial inoculum increases. In this connection, it is of interest to note that there appears to be an inordinate decrease in drug effectiveness against the larger inoculums. Furthermore, the drugs' activity ratio for 39:37°C. is much less for the heavy than for the light inoculums. This would suggest that the activity exhibited by large amounts of drug against large inoculums of bacteria may be non-specific.

Although the temperature effect reported in this paper may not be directly concerned with the question of the *mode* of action of sulfonamide-type drugs, it may have a significant bearing upon

the *definition* of such action. A survey of the literature on this subject indicates that there is considerable disagreement as to whether these drugs exhibit merely an inhibitory, or a fully bactericidal action *in vitro.*

Thus, bactericidal action has been reported by Colebrook and his associates (1936); Helmholtz and Osterberg (1937); Hoare (1938); Keefer and Rantz (1938); Mayer (1937); Mellon and Shinn (1937); Neter (1938); Nitti and his associates (1937); Wengatz and his associates (1938); and White and Parker (1938). On the other hand, bacteriostatic action has been reported by Bliss and Long (1938); Gay and Clark (1937); Long and his associates (1939); Lyons and Mangiaracine (1938); Maegraith (1938); Osgood and Brownlee (1938); Mellon and Bambas (1937); and Spray (1938). In a recent report (1939), McIntosh and Whitby have made an unqualified statement to the effect that sulfanilamide is inactive against streptococci in peptone-containing broth.

It is obvious that these conflicting results must be explained before one can define the *in vitro* action of the drugs. Differences in drug concentration, bacterial strain, test medium, or size of bacterial inoculum probably account for many of the discrepancies. However, even under the most favorable conditions for reproducibility, it is evident that contradictory results may be obtained, if maintenance of accurate temperature control is neglected. For example, tests made in two different laboratories, to determine the activity of 10 mgms. per cent sulfanilamide against a small inoculum of strain C 203 in PD broth, might show contradictory results, varying between no effect and sterilization, if incubation were carried out at 36°C. in one laboratory and at 38°C. in the other. Therefore, in view of the relatively inaccurate temperature control which one finds in the ordinary bacteriological incubator, it is suggested that more precise incubation temperature may be essential to a clear definition of the *in vitro* action of these drugs.

A possible correlation between the action of sulfanilamide at "fever temperature" *in vitro* and its clinical use has been suggested in our previous report (White and Parker, 1938). The present

data, on the action of sulfapyridine *in vitro*, suggests that there may be a similar correlation for this drug. In this connection, it is interesting to note that clinically significant concentrations of both drugs were active only at temperatures above 37°C. A comparison of our sulfapyridine and sulfanilamide data indicates that the average *in vitro* activity ratio for these compounds is about 4.5, in favor of the former. This may afford further opportunity for correlation between our observations and clinical results.

SUMMARY

The minimal bactericidal concentrations of sulfanilamide and of sulfapyridine for each of 6 different initial concentrations of beta-hemolytic streptococcus strain C 203 in PD broth were determined at various temperatures between 30° and 39°C. Incubation of drug-bacteria mixtures was maintained for 48 hours in water baths with thermoregulator control to ±0.005 of a degree. Simultaneous titration of drug and culture, each against the other, with sterilization as the end-point for measuring drug activity, was carried out in a total of 312 determinations.

CONCLUSIONS

Under the conditions of our experimental procedure:

1. Concentrations of sulfanilamide lower than 1000 mgm. per cent were inactive at 30°C.
2. Concentrations of sulfanilamide lower than 100 mgm. per cent were inactive at 36°C.
3. At 39°C., concentrations of 10 mgm. per cent or less were bactericidal against an average initial bacterial concentration of 50,000 per ml.
4. About 100 times as much sulfanilamide was required to sterilize at 37°, as at 39°C.
5. Drug concentrations of 10 mgm. per cent or less were active only at temperatures above 37°C.
6. Sulfapyridine was similarly influenced by temperature, but at a higher level of activity.
7. The average activity ratio for these compounds was 4.5, in favor of sulfapyridine.

8. Accurate temperature control appears to be an essential part of any reliable method for measuring the activity of these drugs *in vitro*.

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FACTORS LIMITING BACTERIAL GROWTH

VII. Respiration and Growth Properties of Escherichia coli Surviving Sublethal Temperatures

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In earlier papers of this series (Hershey, 1939b) it has been shown that growth during the latent period is continuous with the subsequent growth accompanied by cell division, the two phases being described by a single law. The observations were made with *Escherichia coli* and *Shigella dysenteriae*. Further test of the general validity of the conclusions reached with these organisms seemed desirable. This could be done by applying similar methods at random to the study of various species. We have attempted to proceed more purposefully.

One could, *a priori*, anticipate several causes of lag (Penfold, 1914) which would be different from what we shall call the "normal latency" exhibited by the organisms mentioned. For example, growth on transplant should occur at increasing rate if bacteria in an aging culture sustain injury to labile constituents of catalytic mechanisms which are not essential to viability, but on which the rate of growth under favorable conditions depends. No authenticated instance of this is known. Recovery from injury has been suggested as a possible explanation of lag in pneumococcus cultures (Chesney, 1916), which are known to accumulate hydrogen peroxide in toxic concentrations; a similar explanation might be put forward to account for "dormancy" (Burke, Sprague and Barnes, 1925) of spores or of vegetative cells, whether the injury is of unknown cause or wilfully inflicted (Hollaender and Duggar, 1938; earlier work discussed by Buchanan and Fulmer, 1928). It is evident that this mechanism is

not involved in normal latency, but that it may be a contributing factor in special cases.

In seeking experimental verification of this hypothesis, it has seemed preferable to use bacteria subjected to artificial treatment which could be controlled, rather than to observe cultures in which only the effects of uncertain influences could be measured. Our observations have been made with *E. coli* surviving exposure to heat. The general conclusions drawn from these experiments ought to be equally pertinent to an understanding of the effects of hydrogen peroxide, adverse pH, or other change in the medium accompanying the growth of bacteria themselves, should significant injuries be shown to result. The following experiments are concerned, therefore, with the measurement of delay in multiplication following physical injury, and with the elucidation of the physiologic significance of this phenomenon. In addition, information has been obtained relating to the interdependence of respiratory changes and loss of viability in bacteria subjected to various degrees of heat.

EFFECTS OF HEAT ON VIABILITY

The systematic observations of Curran and Evans (1937) showed that serious errors are introduced when the usual pour-plate method is used to count injured bacteria. By adding suitable enrichments to the agar medium, much higher survivor counts were obtained. It is a truism that the viable count must be expressed in terms of ability to grow on some arbitrarily selected nutrient medium. But for the purpose of studying growth properties, the significant count should represent the number of cells which will eventually undergo multiplication in the culture under observation. The obvious method of obtaining this number is to employ for counting the medium in which growth is to be observed. This is conveniently accomplished, with liquid media, by the method of most probable numbers (McCrady, 1918).

By this method it was found, with bacteria surviving the higher temperatures, that the numbers given by the plate count were too low (table 1), sometimes representing only one per cent of the

numbers growing in broth. During incubation in broth, however, the number of organisms capable of growing in agar rapidly rose to the values given by the direct broth count (fig. 2). Since a constant value was reached well in advance of the actual multiplication of bacteria, it was possible to take advantage of the greater convenience of the plate count in measuring the length of the latent period. One or two counts by the method of probable numbers at the beginning of each experiment sufficed as controls.

It is evident that the explanation of Curran and Evans, that survivors exhibit altered nutrient requirements, is not supported

TABLE 1

Respiratory activity and viable bacteria surviving 15 minutes exposure at different temperatures

EXPERIMENT	TEMPERATURE	NUMBERS BEFORE HEATING × 10^9/ML.	SURVIVORS (BROTH) × 10^9/ML.	PER CENT NON-VIABLE (BROTH)	SURVIVORS (AGAR) × 10^9/ML.	PER CENT NON-VIABLE (AGAR)	O_2-USE* BEFORE HEATING MM³/ML. HR.	O_2-USE* AFTER HEATING MM³/ML. HR.	PER CENT DECREASE O_2-USE
	°C.								
1	51.0	3.5			3.5	0	327	219	33
2	52.0	4.5	5.0	0	4.0	11	257	38	85
3	52.5	3.5	4.0	0	3.6	0	234	28	88
4	53.5	4.8	3.9	19	2.3	52	302	3.3†	>99
5	54.0	3.6	2.9	19	1.8	50		0.93†	>99
6	56.0	4.0	0.61	85	0.002	>99		0.00027†	>99

* At zero time.
† By extrapolation.

by these observations, since the broth and agar used were of the same nutrient composition. A few trials showed that the low plate counts were not due to the effects of the temperature of the melted agar, for broth tubes heated after seeding at 48° for several minutes did not give low counts. The failure of viable cells to form colonies in agar could be attributed to the effect of agar itself in decreasing the available water content of the medium, since adding small quantities of water to each plate increased numbers of colonies. The recovery of ability to produce colonies in agar resembled other growth processes in that it did not occur in broth at low temperatures, nor in saline at 37°C. These

observations probably explain the "apparent initial increase" during lag noted by Hollaender and Duggar (1938) with irradiated bacteria.

EFFECT OF HEAT ON LATENT PERIOD

Bacteria surviving heat showed a progressively lengthened period of latency as the temperature was increased. At 56°C.

TABLE 2

Observed and calculated latent period and bacterial numbers in cultures seeded with heated bacteria

EXPERIMENT	TEMPERATURE, 15 MIN.	S_0 $\frac{MM^3 \times 10^{-7}}{CELL\ HOUR}$	L OBS.	L EQ. (2)	LOG B_0‡		LOG B‡ OBS.	LOG B EQ. (5)
	°C.		*hours*	*hours*		*hours*		
1	51.0	0.63	1.6	1.65	9.54	2.50	10.84	10.46
2	52.0	0.085	2.4	2.58	9.65	4.00	11.60	11.09
3	52.5	0.083	2.5	2.59	9.54	4.00	11.26	10.97
4	53.5	0.0085*	3.2	3.66	9.59	4.40	10.91	10.41
5	54.0	0.0032*	4.1	4.12	9.46	5.66	11.03	11.02
6	56.0	0.0000044*	7.9	7.07	8.79	9.00	9.90	10.60
7	Unheated	0.85†	1.45	1.51	9.99	2.00	10.73	10.57
8	Unheated	0.85†	1.53	1.51	9.93	2.00	10.42	10.51
9	Unheated	0.85†	1.53	1.51	9.72	2.25	10.66	10.54

* By extrapolation, see "Methods."

† Average value previously reported.

‡ By count from subcultures containing approximately 10^4/ml., computed to numbers per ml. original culture.

$$\text{Eq. (2)}: L = \frac{\log 4S/3S_0}{M \log 2}$$

$$\text{Eq. (5)}: \log B = \log B_0 + Mt \log 2 - \log S/S_0.$$

$$S = 16.1 \times 10^{-7} \text{ mm}^3 \text{ per cell hour.}$$

$$M = 3.1 \text{ doublings per hour.}$$

for 15 minutes, for example, the survivors numbered 15 per cent of the original cells, and reached the midpoint of the first generation only after 8 hours in broth at 37°C. as compared with 1.5 hours for the control (table 2). Our data are apparently the first to show clearly that this extended latency is a direct effect of injury and not merely the result of selection of resistant individuals inherently slower to develop, since delayed multiplication was noticeable after exposure to temperatures at which no

death occurred. Previous workers have observed similar extension of latent period only under conditions in which the percentage of survivors was small (Hollaender and Duggar, 1938; other work discussed by Buchanan and Fulmer, 1928). This discrepancy is only partially explained by the fact that different criteria of survival were used.

EFFECT OF HEAT ON RESPIRATION

The decrease in rate of oxygen consumption, at zero time in broth, of cells exposed to various temperatures was enormously

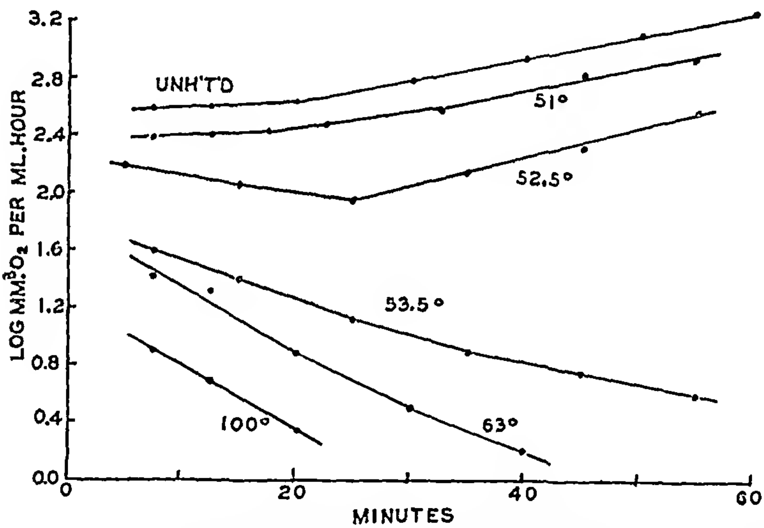

FIG. 1. RATES OF OXYGEN-CONSUMPTION IN BROTH OF E. COLI AFTER 15 MINUTES EXPOSURE TO DIFFERENT TEMPERATURES

Each vessel contains 0.4 to 2.0 ml. cells, 3.6 to 2.0 ml. saline, and 1.0 ml. solution containing 10 per cent peptone, 5 per cent beef extract, and M/10 phosphate pH 6.8, the latter added at zero minutes. Cells consist of 24 hour aerated broth culture, 1 ml. containing 2.5 to 5.0 billion organisms.

greater than the accompanying decrease in numbers. Heating to 52.5°C. for 15 minutes depressed oxygen use about 90 per cent without killing any of the cells. Their ability to grow in agar was somewhat decreased however (table 1). Actual curves showing initial rates of oxygen consumption in broth for several experiments are given in figure 1. It will be observed that following exposure to the higher temperatures, and even with

completely sterile suspensions, there was a residual respiration which decreased very rapidly for a time after addition of fresh broth, soon becoming immeasurable if sufficient heat-injury was sustained by the cells. The same peculiarity, even more marked, was observed in a few measurements of carbon dioxide produced at pH 6.6 by the heated bacteria, i.e., the ratio CO_2 produced to

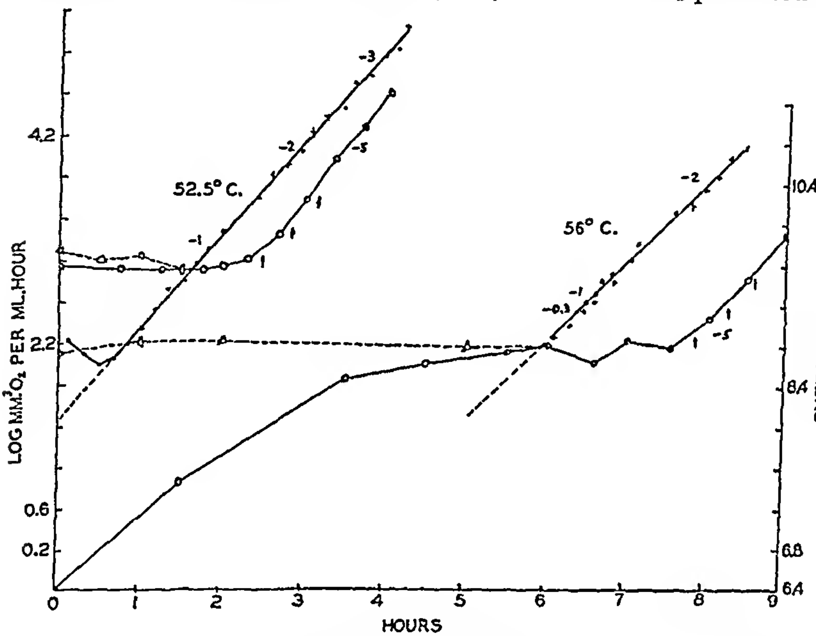

FIG. 2. MANOMETRIC AND POPULATION GROWTH CURVES FOR CULTURES SEEDED WITH HEATED BACTERIA

Experiments with bacteria surviving 52.5 (experiment 3) and 56.0 (experiment 6) degrees C. Viable counts by method of probable numbers (broken lines) and agar plates. Arrows indicate mid-points of successive generations. Manometric data from measurements of O_2-consumed by successive tenfold dilutions made in broth at zero time, and incubated in respirometer vessels without KOH until readings were begun. Vessels contain 4 ml. aliquots of dilutions indicated by negative logarithms.

O_2 absorbed tended to decrease as the rate of respiration decreased. The phenomenon was observed in the same degree whether measurement was made soon or late after the cells were heated, and could be elicited a second time by adding a second portion of broth. One may suppose that the broth contains small amounts of substrates for which the corresponding activating centers are relatively heat resistant.

For obvious reasons, this evanescent respiration has been ignored in arriving at the values recorded in the table (see fig. 2). For decreases greater than 90 per cent, the latter are hypothetical, since extrapolation to zero time required assumptions that cannot be proved. It was observed that the rate of oxygen consumption increased logarithmically from zero time when measurable, i.e., when 10 per cent or more of the original activity remained. Following exposure to the higher temperatures, increase in respiration was of the same kind during the latter part of the latent period, as soon as it could be measured. In extrapolating through the unobserved period, it was consistent to assume that the same acceleration had prevailed from the beginning of the experiment. To the extent that this assumption is questionable, the observations with cells heated at the higher temperatures are less satisfactory from an experimental point of view.

RELATION BETWEEN RESPIRATION AND GROWTH

In the work with bacteria obtained under various conditions of cultivation (Hershey, 1939b), we have often used "oxygen consumed per cell-hour" as a measure of physical size, the relationship between these two properties appearing to be constant (Hershey and Bronfenbrenner, 1938). With heated bacteria, it is clear that this relationship no longer holds, since heating may affect respiration but not size. Only the manometric method, therefore, could possible give physiologically significant values of S_0, which accordingly must be redefined as "functional size," i.e., initial rate of O_2-consumed per cell-hour, without other implication.

With this reservation, laws describing growth at constant rate apply without modification to bacteria surviving heat (table 2). In other words the regeneration of heat-inactivated cellular respiratory mechanisms occurs at the same rate, and reaches the same maximal value preceding cell-division, as is the case with unheated cells (fig. 2). On the other hand, since the rate of increase measured manometrically is continuously proportional to the actual rate of O_2-consumption, but not initially to the total

amount of heated cell substance, it is evident that the growth of heat-treated cells occurs in accordance with the classical notion of lag. From the standpoint of cell physiology, however, this

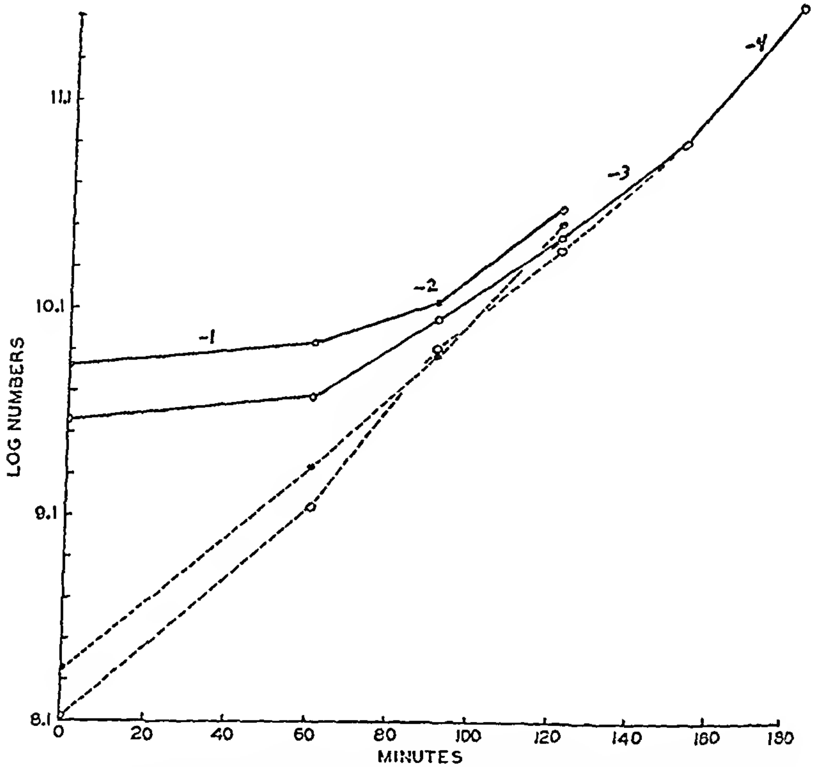

FIG. 3. GROWTH IN TURBIDITY DURING THE LATENT PERIOD OF CULTURES CONTAINING HEATED BACTERIA, SHOWING THAT RECOVERY IS ACCOMPANIED BY PHYSICAL GROWTH

Two experiments are shown with cells heated 15 minutes at 52.0 and 52.5°C. respectively. All the cells are viable. The upper curves represent the observed turbidity in terms of the calibration of the instrument. The lower curves were obtained by subtracting, from the observed values, nephelometric equivalents of the initially inactive respiratory enzymes (see "methods"). As before, counts made from the subcultures are expressed in terms of numbers per ml. of the original cells. Dilutions of the subcultures are indicated on the curves by negative logarithms.

behavior is best described as that of a very small cell exhibiting normal growth properties, but burdened with a relatively large mass of inert protoplasm. This relationship is clearly shown in figure 3. The upper curves represent the observed increase in

turbidity of cultures of heated bacteria, and exhibit true lag. Points on the lower curves were obtained by subtracting from each nephelometric value a constant representing the amount of inert protoplasm initially present, that is to say, the initial nephelometric count multiplied by the fractional decrease of oxygen consumed per cell-hour at zero time. No cell death occurred at the temperatures chosen.

The latter experiments answered a question of particular interest: Is the regeneration of heat-inactivated respiratory function quantitatively accompanied by physical growth? The identity of the corrected nephelometric curves with the analogous manometric curves (fig. 2 and 3) indicates that it is. The conclusion seems warranted that increase of respiratory activity and recovery of ability to produce colonies on agar (see page 565) during the latent period of heated bacteria in broth are effects of actual physical growth, and that this growth is "normal" in that it occurs at a rate proportional to the amount of surviving respiratory function, or of some unidentified cell function of which the former is a quantitative measure. It does not appear that any reactivation of metabolic properties of the inert cell residue takes place.

If regeneration of respiratory function regularly occurs at the normal growth rate, as indicated by the experiments cited above, population growth in cultures seeded with heated bacteria ought to be predictable in terms of growth at constant rate to constant maximal size preceding cell division. Conversely, agreement between observed and expected numbers in these cultures should provide an independent confirmation of the conclusions already reached.

Growth data obtained by the viable count with bacteria heated at various temperatures are compared with the corresponding values calculated by our equations describing growth without lag (Hershey, 1939b) in table 2. The experimental values of L (latent period) and log B (numbers at any time during the period of rapid cell division) were measured as previously described. In the present experiments L was determined less accurately because it was impracticable to make closely-spaced counts during the

extended latent periods under observation. This value is probably correct within a half-hour. Growth curves from two experiments are reproduced in figure 2. The experimental data of table 2 include all the measurements of L made, and the values of log B representing the last count made during each experiment. Repeated manometric experiments were necessary to establish within experimental variation a constant value for the growth rate (M) during the latent period. The curves of figure 2 (M = 3.0 and 2.6) show the considerable variations met with. The average, 3.1, of values ranging from 2.6 to 3.3, was used in all calculations. This is, of course, also the normal rate of growth of this organism. Since the data under consideration are unselected, the agreement between theory and experiment may be regarded as satisfactory.

GENERAL DISCUSSION

The observations reported in this paper seem important to us in several connections. If our interpretation is correct, they establish the physiological identity of the extended latency of heated bacteria, and the period of lag exhibited on transfer by unheated cultures. In both cases, the period preceding cell-division consists of growth at a characteristic maximal rate not different from that seen in rapidly multiplying cultures.

It is suggested that the ideas of injury and repair do not describe the effects of heat on bacterial cells. On the contrary, the detectable effects appear to be quantitative only, both with respect to respiration and growth properties. Since the growth of surviving cells is not different from normal growth, the term repair is misleading. The distinction is perhaps trivial, but is consistent with our objection (Hershey and Bronfenbrenner, 1938) to applying the concepts of mammalian physiology too uncritically to the physiology of undifferentiated cells. In other connections this habit has had the practical consequence, we believe, of prompting misguided efforts.

The significance of the experimental value "oxygen consumed per cell-hour" appears to be manifold: under suitable conditions it is a measure of cell-size; with both normal and heated bacteria

it provides an index of growth function; within a critical range it appears to be a factor conditioning viability. It gives point to the relative meaning of the latter concept: within a critical range of values of "O_2-consumed per cell-hour," viability is the expression of the probability[1] that a given quota of respiratory activity will furnish sufficient energy for growth in a given environment. The more favorable is the latter, the lower will be the critical metabolic rate coinciding with loss of ability to propagate.

We do not, of course, suggest that the respiratory mechanisms are the sole determinants of growth. Under the conditions we have studied, there is a constant quantitative relation between respiratory activity and rate of growth. We conclude that either the activity of energy producing systems under these conditions is the limiting factor in the sequence of processes recognized as growth, or that the synthetic mechanisms themselves are capable of quantitative variations in activity which are by coincidence measurable in terms of oxygen consumed per cell. It should be recalled that these "synthetic" mechanisms do not appear to be demonstrable independently of growth and respiration.

A quantitative definition of viability is pertinent to the problem of the kinetics of death. Loss of viability, whatever the criterion used, is an all-or-none result, probably representing in terms of altered cell function the 90 per cent or more completion of some continuous process, exemplified by progressive inactivation of respiratory enzymes. It is to be anticipated that measurement of the course of this process[2] in time will provide a more satisfactory description of injury sustained than measurement of numbers surviving, and the kinetics of the two results will be

[1] Rahn (1929–30) has treated the process of death in bacterial populations by the probability method. He postulated a number of vital "genes," inactivation of any one of which would lead to the death of the bacterial cell. But since the available data on the kinetics of death would be very different if other media had been used for counting, they are scarcely to be interpreted in terms of single vital molecules.

[2] We hope to be able to report, in the near future, on a method for measuring this quantity which requires no assumption as to the nature of the processes involved.

very different. Rahn and Barnes (1932–33), and Oster (1934–35) have given suggestive data of analogous kind obtained with yeast.

The present work provides circumstantial evidence that the cell may be regarded as the sum of its metabolic activities, and more specifically, that the rate of growth is limited by the rate at which energy is degraded by the cell. Equally plausible is an alternative interpretation: that the observed correlation is the accidental result of simultaneous inactivation of energy-producing and synthetic mechanisms by heat. If the latter is true, study of different types of injurious influences ought to lead to different quantitative results. Pertinent observations made by Bronfenbrenner and collaborators (1939) with disinfectants are in superficial agreement with the experiments using heat. We intend to pursue this question with a more detailed study of the relation between effects of phenol on respiration, and on growth properties.

METHODS

Details of experiments employing most of the present methods have been given in earlier papers (Hershey 1939a and b). The cultures used had been incubated with aeration at 37°C. for 24 hours. Samples of these were heated in test tubes in a temperature controlled water bath.

Figure 1 is self-explanatory. The curves shown are not necessarily those corresponding with the experiments of tables 1 and 2. In arriving at zero time values from curves of this kind, the straight portion of the graph was extrapolated to the ordinate. This procedure was adopted since some decision was necessary in the case of heated bacteria if the momentary period of decrease in respiration was to be ignored. The method used has the virtue of objectivity. Actually it gives figures somewhat too low, as indicated by values recorded in table 1 for unheated culture. These would give S_o in the neighborhood of 0.6, while extrapolation through the observed points has yielded an average value of 0.85. This probably accounts for the tendency of calculated values of L to be too large, and of log B too small, in the present

experiments. By this procedure the curves of figure 1 obtained with cells heated at 53.5° and above are left out of calculations entirely. In defense of this practice, it may be pointed out that we are defining S_o as O_2-consumed per viable cell, and attempting to give it physiological significance; the data of figure 1 would give infinite values with non-viable cells (63 and 100°C.). In this sense it is consistent to regard all decreasing initial rates as imaginary. For other reasons, the initial values obtained with cells heated at temperatures above 53° are of little quantitative significance. In order to obtain measurable rates, relatively large numbers of heated bacteria must be used. Under these conditions the observed oxygen consumption is not strictly proportional to numbers of cells, and varies somewhat with the concentration of broth. This is probably explained by the fact, previously referred to, that the substrates principally oxidized by these cells are present in low concentrations in the broth. Unheated cells, on the other hand, probably utilizing at measurable rate many more substrates, give constant experimental values for O_2-consumed per cell over relatively wide ranges of cell and broth concentrations, the limiting factor in this case being solubility of oxygen (Hershey and Bronfenbrenner, 1937). These facts should not be interpreted as evidence of altered metabolic requirements of heated cells, except in the sense that the rates of inactivation of the various cell catalysts are different (Quastel, 1928–29). The method of extrapolation actually used is shown in figure 2. Again the logarithmic portion of the curve is extended to zero time, either graphically (experiment 3), or where this procedure would be too arbitrary (experiment 6), by the use of the formula

$$M = \frac{\log R_{O_2} - \log x}{t \log 2}$$

where $M = 3.1$ doublings per hour, R_{O_2} = rate of O_2-use observed at time t, x = corresponding rate at $t = O$. Values of x (O_2-use after heating, table 1) for experiments 4, 5 and 6 are the average of solutions for different values of t. As stated earlier, the experiments at the higher temperatures are not entirely satisfactory because of the assumptions involved.

Figure 2 also gives examples of population curves for subcultures of heated bacteria, showing the use of the method of most probable numbers in enumerating survivors, and illustrating the fictitious results given by plate counts during the early hours of growth with bacteria heated at the higher temperatures.

Figure 3 requires no description, except for the method of computing values for the corrected curves. For example, photoelectric analysis of heated cultures, using the instrument of Stier, Arnold and Stannard (Hershey, 1939a), indicated 6.6×10^9 cells per ml. But "O_2-consumed per ml." for the same preparation, divided by the nephelometric count, gave 0.085×10^{-7} mm.^{3} per nephelometric cell per hour, whereas unheated cells, since the instrument is calibrated against 24-hour stationary cultures, should give an average value of 2.4×10^{-7} mm.^{3} per cell hour (*Hershey and Bronfenbrenner, 1938;* see correction *Hershey,* 1939b). Therefore, $6.6 \times 10^9 \times 0.085 \div 2.4 = 2.3 \times 10^8$, the nephelometric equivalent of the initial O_2-consumed per ml., and $6.6 - 0.23 = 6.37 \times 10^9$, the nephelometric equivalent per ml. of original cells of the inactivated respiratory function. Points on the lower curves of figure 3 were obtained by subtracting this quantity from the corresponding points on the experimental curves, these being expressed in terms of the ml. of original cells. Numerous sources of error in these experiments make the exact values questionable. It must be accepted that physical growth occurs during the period of recovery; in addition the corrected curves appear to be "logarithmic."

"Numbers before heating" (table 1) are the result of a single plate count made from the unheated culture. "Survivors" in broth are the averaged results of counts made by the method of most probable numbers (McCrady, 1918). Since lactose-brom-thymol-blue broth gave the same count as plain broth with heated bacteria in this method, the former was used to facilitate detection of contaminants. Ten tubes were seeded with each dilution required for each count made. Agreement between duplicate counts was usually satisfactory for our purpose with this number of tubes, although 100 per cent variations were occasionally met with. "Survivors" in agar are the result of a

single plating made immediately after heating the cells. These counts were relatively inconsistent, plates receiving the same aliquot, and especially those receiving different volumes of the saline dilutions (see page 565) giving very poor agreement.

Values of B_o (table 2) represent the average of the several plate counts showing agreement immediately before multiplication began (fig. 2). The observed latent period (L) was obtained from the growth curves as previously described (Hershey, 1939b). Values of log B observed, to avoid selection, were taken from the last count made in each experiment. All values required for solution of equations (2) and (5) are given in table 2.

SUMMARY

When *Escherichia coli* is subjected to heat, three effects may be recognized: decreased respiration in broth, prolonged latent period on subculture, and finally, loss of viability. The correlation between these various effects is discussed at length.

The regeneration of heat-inactivated respiratory function occurs at a constant rate characteristic of the normal growth of the organisms, and is accompanied by physical growth. "Recovery from injury" appears, therefore, to consist of growth.

The extended latent period of heated cells answers to the classical description of lag. In terms of cell physiology, however, it is not different from the latency of unheated bacteria, and consists of growth at constant rate.

Equations describing growth at constant rate satisfactorily predict the growth properties of heated bacteria, if quantitative changes in cell respiration are taken into account.

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STUDIES ON IMMUNIZING SUBSTANCES IN PNEUMOCOCCI[1]

X. The Relationship between the Acetyl Group on Type I Pneumococcus Polysaccharide and Antigenicity[2]

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It has been established by many investigators that pneumococcus polysaccharide, isolated either from the bacterial cell or from the bacteria-free broth culture, is antigenic for mice and men. Hence, the way is clear to determine the relationship between chemical constitution and antigenicity. Some efforts have been made toward the establishment of this relationship. It was the observation of Avery and Goebel (1933) that the antigenicity of the Type I polysaccharide was probably related to the presence of an acetyl group, which prompted this study. Their proof of the presence of this chemical radical was the effect of heat treatment with dilute alkali, accompanied by a loss of antigenicity, and also the isolation of silver acetate from the distillate from an acid solution of the sample which had been treated with alkali and heat. In the meantime, Enders (1930) had reported isolation of his "A" substance, and later (Enders and Wu (1934)) stated that it was the same as the acetyl polysaccharide of Avery and Goebel. Also Sevag (1934), and Heidel-

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berger, Kendall and Scherp (1936) have confirmed this report as regards the presence of the acetyl group on Type I polysaccharide (SSS). The *latter investigators* have also stated that Type II polysaccharide contains an acetyl radical. Inasmuch as these observations if confirmed would constitute an important contribution toward our knowledge of antigens, attempts have been made to corroborate the findings.

Our investigations on the isolation of the immunizing substance of the pneumococcus confirm in great part the work of Schiemann and Casper (1927–28), Saito and Ulrich (1929), Wadsworth and Brown (1933), Zozaya and Clark (1933), and others, that a substance polysaccharide in nature is antigenic for white mice (Felton 1935)). In 1928, as stated in a paper on the dissociation of antigen-antibody complex (Felton 1932)), was begun the use of the calcium phosphate method for isolating polysaccharides (Felton 1935)). The method has been continued since because of the high antigenicity of the product for white mice, and also as shown in another publication (1931), because the product precipitated practically all of the antibody from immune serum, and differed in this respect from the original preparation of Heidelberger and Avery (1923). At the time Avery and Goebel reported the presence of an acetyl group on the Type I polysaccharide, the effect of alkali, organic and inorganic, was being studied on products isolated by the calcium phosphate method. It was found that while heat and inorganic alkalies, with the exception of ammonium hydroxide, decreased antigenicity, organic bases did not decrease but actually increased this activity (1936). This observation along with other considerations led us to question the importance of the acetyl group on Type I polysaccharide in relation to antigenicity. This paper is a comparative study of the polysaccharide of Type I pneumococci isolated by the calcium phosphate method and that isolated by the revised method of Heidelberger. It includes the effect of alkali and acid, under conditions which deacetylate a possible acetyl group, on the antigenicity of the polysaccharides isolated by these two methods.

METHODS

The methods used were the conventional ones for: nitrogen, glucose number, optical rotation, precipitin titer, immune precipitable nitrogen (Heidelberger, Sia and Kendall, 1930), and active immunity produced in mice. Two methods have been used for this last test, one varying the dose of antigen from 0.5 ml. of 10^{-3} to 0.5 ml. of 10^{-8} dilution in logarithmic series and testing seven days later with 100 to 500 lethal doses of the homologous type of pneumococcus; the other injecting 0.5 ml. 10^{-6} polysaccharide dilution and testing the mice after the seven-day period with variations of 8-hour culture beginning with 0.5 ml. 10^{-3} up to 0.5 ml. 10^{-8} dilution in logarithmic series. Inasmuch as this latter method avoids the zonal effect of the former, it alone has been given in these experiments.

The determination of the acetyl group was carried out by a modification of the Pregl technic. This modification seemed necessary because it was found that by the original method most simple sugars apparently contained an acetyl group as measured by the amount of distillable acid. To obviate this difficulty, a trap was devised which, judging from tests with glucose, prevented the distillation of the acid degradation products of this carbohydrate. The method also gave theoretical values for glucose pentacetate, as well as other non-volatile organic acetates. Moreover, the work of Nef (1914), and of Evans (1926) showed the possibility of breaking up complex sugars into a mixture of various acids by acid or alkali treatment. Thus, it seems imperative that the acid distilled from the various polysaccharide preparations be identified. Three methods have been tried: first, the one used by Avery and Goebel, the silver salt of acetic acid; second, the Reid method (1917) of crystallization with p-nitrobenzylbromide in alcohol; and third, the lanthanum-nitrate iodine—spot test of Krüger and Tschirch (1929, 1930), which is positive only with propionic and acetic acids. Briefly, it may be stated that it has been impossible to isolate silver acetate from any SSS preparations made by the calcium phosphate method. With the Reid method, crystals were obtained with consider-

able regularity but in no case was the melting point found to be 78°C.—some were higher, some lower, varying from 84° to 72°C. Whether these crystals were uniform was not determined, inasmuch as insufficient material was obtained for purification by recrystallization, with one exception in which the melting point of both first and second crystallization products was 72°C. However, the lanthanum-nitrate iodine test for acetic acid was ideal with respect to sensitivity and practicability for the purpose at hand. The technic was the same as reported by the originators, and consisted of the use of 0.05 ml. sample with 0.05 ml. of 5 per cent lanthanum nitrate, 0.05 ml. N/100 iodine solution, and 0.05 ml. normal ammonium hydroxide. The reaction was carried out on the usual cupped spot plate. The distillate from a given sample, generally from 20 mgs. material, was titrated with sodium hydroxide in the usual manner and then evaporated to dryness, and the precipitate dissolved in 0.5 ml. water. This solution was then tested as above indicated. In addition, to rule out a possible inhibitory action, to a 0.05 ml. sample was also added 0.05 ml. of N/100 sodium acetate and a test made, along with a control of the same amount of acetate in water. However, in the preparations tested, no inhibitory action was found. It is pertinent to add that 30 organic acids were tested, and none, with the exception of propionic and acetic as suggested by the authors, gave a positive test. The test is unmistakable as it is a deep blue color depending in intensity on the amount of acetic acid present. Under the conditions of our test, 0.05 ml. of 0.02 normal sodium acetate gave a positive reaction.

EXPERIMENT I

Effect on antigenicity of treatment with ammonium hydroxide

The first experiment shows the influence of ammonium hydroxide (sp. gr. = 0.89) on the antigenicity of two preparations, one made by the calcium phosphate and the other by the Heidelberger method. To 100 mgs. of each dissolved in 100 ml. water was added an equal volume of ammonium hydroxide (sp. gr. = 0.89) at room temperature, and the mixtures held for 18 hours.

The polysaccharide was then precipitated with alcohol and ether, washed thoroughly with alcohol and then with ether, and dried in a vacuum desiccator over calcium chloride. As can be seen from table 1, the nitrogen and the glucose number changed but little with this treatment. However, there was a greater change in the sample made by the Heidelberger method than in the calcium phosphate preparation. (Hereinafter called H and F preparations respectively.) There was an increase in precipitin titer of the F preparation, and a decrease of the H. The optical

TABLE 1a

Effect on antigenicity of treatment with ammonium hydroxide

SAMPLE	NITROGEN	GLUCOSE NUMBER	PRECIPITIN TITER	DISTILLABLE ACID	ACETIC ACID SPOT TEST	OPTICAL ROTATION
	per cent	*per cent*		*per cent*		
P192(F)	4.28	24.00	1:2,500,000	7.53	Neg.	+120
P192(F) + NH_4OH	4.35	24.24	1:5,000,000	1.08	Neg.	+170
P192(H)	3.13	23.40	1:5,000,000	2.69	Neg.	+140
P192(H) + NH_4OH	2.73	21.60	1:2,500,000	1.33	Neg.	+160

Immune precipitable nitrogen

SAMPLE	DILUTIONS OF SAMPLES				
	1:2500	1:5000	1:10,000	1:15,000	1:20,000
P192(F)	0.370	0.302	0.264	0.199	0.160
P192(F) + NH_4OH	0.434	0.306	0.286	0.222	0.173
P192(H)	0.380	0.342	0.304	0.264	0.180
P192(H) + NH_4OH	0.388	0.334	0.266	0.264	0.176

rotation was increased in both preparations. A comparison of the initial preparation of both shows that there was a slight difference in immune precipitable nitrogen of the H over the F; but after treatment with ammonium hydroxide, the reverse was true. The differences to be stressed in this report, however, are the amount of acid distillable under vacuum, the result of a test in this distillate for acetic acid, and the antigenicity of the two preparations. From 20 mgm. of the F preparation, there was 7.5 per cent distillable acid, and of H 2.69 per cent; after treatment with ammonium hydroxide both were decreased to approxi-

mately 1 per cent. However, neither preparation gave a positive test for acetic acid with the Krüger and Tschirch spot test. The antigenicity of the F sample was not altered, and of the H perhaps

TABLE 1b

Effect on antigenicity of treatment with ammonium hydroxide

Mouse immunity against Type I culture

SAMPLE DILUTED 10^{-6}	DILUTIONS OF CULTURE					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
P192(F).................	18*	S	S	S	94	S
	S	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P192(F) + NH_4OH.......	18	S	20	S	S	S
	20	S	S	S	S	S
	46	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P192(H).................	18	24	S	66	24	S
	18	40	S	S	24	S
	18	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P192(H) + NH_4OH.......	18	40	40	90	S	40
	24	90	40	S	S	S
	24	S	S	S	S	S
	40	S	S	S	S	S
	S	S	S	S	S	S
Culture control..........				18	20	20
				18	24	20
				18	24	24

* Numbers refer to hours of survival; S indicates survival.

slightly reduced, with ammonium hydroxide treatment. Thus, it would appear from this experiment that the preparations of SSS by the two methods yielded a product with no demonstrable acetyl group and yet with high antigenic activity in white mice.

EXPERIMENT II

Effect on antigenicity of treatment with sodium hydroxide and ammonium hydroxide at room temperature for 24 hours

In the second experiment, the effect of normal sodium hydroxide and of ammonium hydroxide (sp. gr. = 0.89) is compared on two preparations made by the calcium phosphate and the

TABLE 2a

Effect on antigenicity of treatment with sodium hydroxide and ammonium hydroxide at room temperature for 24 hours

SAMPLE	NITROGEN	GLUCOSE NUMBER	PRECIPITIN TITER	DISTILLABLE ACID	ACETIC ACID SPOT TEST	OPTICAL ROTATION	YIELD
	per cent	*per cent*		*per cent*			*per cent*
P191(F)	3.64	9.96	1:5,000,000	1.83	Neg.	+210	
P191(F) + N NaOH	4.01	6.84	1:5,000,000	1.40	Neg.	+210	85
P191(F) + NH_4OH	3.81	7.08	1:5,000,000	0.99	Neg.	+240	80
P194(H)	3.08	23.64	1:5,000,000	4.52	++	+200	
P194(H) + N NaOH	2.75	8.40	1:5,000,000	0.65	Neg.	*	84
P194(H) + NH_4OH	3.58	21.00	1:5,000,000	1.72	+	*	80

Immune precipitable nitrogen

SAMPLE	DILUTIONS OF SAMPLES				
	1:2500	1:5000	1:10,000	1:15,000	1:20,000
P191(F)	0.308	0.292	0.286	0.286	0.238
P191(F) + N NaOH	0.396	0.336	0.294	0.284	0.286
P191(F) + NH_4OH	0.360	0.364	0.354	0.322	0.284
P194(H)	0.540	0.540	0.392	0.350	0.320
P194(H) + N NaOH	0.360	0.360	0.248	0.236	0.230
P194(H) + NH_4OH	0.334	0.318	0.318	0.310	0.314

* Insufficient sample.

Heidelberger methods respectively. The same general technic was carried out as in the previous experiment. Significant differences in the two original untreated preparations may be seen in table 2. The F preparation was low in glucose number, in distillable acid, and in immune precipitable nitrogen, as compared to the H preparation. It would also appear, inasmuch as the spot test for acetic acid was positive, that the H preparation

contained acetyl groups: the original was positive, the one treated with normal sodium hydroxide at room temperature negative, and the one with ammonium hydroxide positive. It

TABLE 2b

Effect on antigenicity of treatment with sodium hydroxide and ammonium hydroxide at room temperature for 24 hours

Mouse immunity against Type I culture

SAMPLE DILUTED 10^{-6}	DILUTIONS OF CULTURE					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
P191(F)	S*	S	40	44	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P191(F) + N NaOH	18	S	S	S	S	S
	40	S	S	S	S	S
	S	S	S	S	S	S
P191(F) + NH_4OH	18	S	S	S	S	S
	18	S	S	S	S	S
	S	S	S	S	S	S
P194(H)	18	40	S	S	S	S
	18	S	S	S	S	S
	18	S	S	S	S	S
P194(H) + N NaOH	18	18	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P194(H) + NH_4OH	S	40	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
Culture control				18	18	22
				18	18	22
				20	22	24

* S indicates survival; numbers refer to hours of survival.

should be pointed out however that the H method of preparation utilizes a high concentration of sodium acetate, and there is a possibility of residual acetate absorbed on or mixed with the polysaccharide, sufficient to give at least a positive spot test.

This individual preparation was not purified by dialysis as a last step; but one other sample made by the H method was so purified, with the result that the acid distilled from vacuum gave a negative test for acetic acid. However, it is not our intention at this time to prove the presence of acetyl groups on the polysaccharide prepared by the Heidelberger method, but rather to show that, if present, the acetyl group is not essential for the production of active immunity in white mice. This seems to be definitely shown in table 2b. In the first place, the original preparation by the F method contained no acetyl groups, and yet was equal if not superior in antigenicity to the sample prepared by the Heidelberger method. In the former, both sodium hydroxide and ammonium hydroxide seemed to decrease antigenicity slightly, while in the latter there was no apparent change in the sample treated with sodium hydroxide from which the acid distilled gave a negative test for acetic acid. On the other hand, in the case of the two original samples, the amount of protein precipitated differed significantly (F, 0.308; H, 0.540). However, after treatment with sodium hydroxide and ammonium hydroxide this activity was approximately the same in both. This is a confirmation of the observations of Heidelberger, and Avery and Goebel, that treatment with alkali decreases the antibody precipitating activity of Type I polysaccharide.

EXPERIMENT III

Effect on antigenicity of treatment with phosphoric acid and with p-toluene sulfonic acid at 100°C.

A more definite proof that the acetyl group is not essential for antigenicity of Type I polysaccharide is shown in this experiment in which a study was made of the two preparations after hydrolysis with the same amount of acid used for the saponification of the acetyl group. Fifty milligram samples of each were hydrolyzed with 1 ml. 5 per cent phosphoric acid, and also with 1 ml. 5 per cent p-toluene sulfonic acid, by heating for 20 minutes at 100°C. The acid solution was then precipitated with alcohol and ether, this precipitate dissolved by addition of sufficient sodium hydroxide to bring hydrogen ion concentration to neu-

trality, reprecipitated with alcohol, and dried. The H sample was chosen because the distillate from it gave a positive test for acetic acid. In table 3, it is seen that the initial preparations were very much alike as to nitrogen, glucose number, precipitin titer, and amount of distillable acid, but differed in that the one gave a negative test for acetic acid and the other a positive test.

TABLE 3a

Effect on antigenicity of treatment with phosphoric acid and with p-toluene sulfonic acid at 100°C.

SAMPLE	NITROGEN	GLUCOSE NUMBER	GLUCOSE NUMBER BEFORE HYDROLYSIS	PRECIPITIN TITER	DISTILLABLE ACID	ACETIC ACID SPOT TEST	OPTICAL ROTATION
	per cent	*per cent*	*per cent*		*per cent*		
P191A(F)	3.68	33.36	0.60	1:2,500,000	4.69	Neg.	+100
P191A(F) + H_3PO_4	4.86	35.04	0.84	1:5,000,000	0.39	Neg.	+80
P191A(F) + p-tol. sulfonic acid	4.51	34.80	0.96	1:5,000,000	0.22	Neg.	+90
P194(H)	3.08	23.64	1.44	1:2,500,000	4.52	++	+200
P194(H) + H_3PO_4	2.88	19.92	1.80	1:5,000,000	0.52	Neg.	
P194(H) + p-tol. sulfonic acid	3.18	22.56	0.0	1:5,000,000	0.65	Neg.	

Immune precipitable nitrogen

SAMPLES	DILUTIONS OF SAMPLES				
	1:2500	1:5000	1:10,000	1:15,000	1:20,000
P191A(F)	0.526	0.426	0.262	0.242	0.187
P191A(F) + H_3PO_4	0.342	0.322	0.228	0.222	0.186
P191A(F) + p-tol. sulfonic acid	0.314	0.258	0.248	0.228	0.156
P194(H)	0.540	0.540	0.392	0.350	0.320
P194(H) + H_3PO_4	0.342	0.314	0.302	0.250	0.220
P194(H) + p-tol. sulfonic acid	0.364	0.348	0.314	0.264	0.236

On the other hand, after saponification, all preparations yielded a small amount of distillable acid, yet all gave a negative test for acetic acid. The amount of immune precipitable nitrogen in the initial preparations again was somewhat less with the F preparation than with the H; but after saponification, both preparations were essentially the same in this respect. On the other hand, no difference was observed in the antigenicity of these

preparations before and after saponification with acid in the amount customarily used for deacetylation. That the amount of acid and the conditions for saponification were adequate is

TABLE 3b

Effect on antigenicity of treatment with phosphoric acid and with p-toluene sulfonic acid at 100°C.

Mouse immunity against Type I culture

SAMPLE DILUTED 10^{-6}	DILUTIONS OF CULTURE					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
P191A(F)	18*	20	S	S	S	S
	40	S	S	S	S	S
	S	S	S	S	S	S
P191A(F) + H_3PO_4	18	20	90	S	S	S
	18	S	S	S	S	S
	66	S	S	S	S	S
P191A(F) + p-tol. sulfonic acid	S	22	20	40	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
P194(H)	18	S	S	S	S	S
	18	S	S	S	S	S
	S	S	S	S	S	S
P194(H) + H_3PO_4	18	20	66	S	S	S
	22	S	S	S	S	S
	S	S	S	S	S	S
P194(H) + p-tol. sulfonic acid	18	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
Culture control				18	20	20
				20	20	40
				20	22	40

* Numbers refer to hours of survival; S indicates survival.

shown by the fact that the H preparation which gave acetic acid originally in the distillate was negative after treatment with either phosphoric or p-toluene sulfonic acid.

DISCUSSION

The issue in this report is the significance of the acetyl group on the Type I pneumococcus polysaccharide in relationship to antigenicity. The study has been a comparison of preparations isolated by the calcium phosphate method and by the revised method of Heidelberger. To date, seven preparations have been made by the Heidelberger technic each from 18 to 36 liters of broth; three of the preparations gave a negative test for acetic acid and four positive. The amount of distillable acid varied from 2 to 7 per cent. At no time was the acetyl group demonstrated on preparations made by the calcium phosphate method; while Heidelberger has reported the existence of this group on the SSS as prepared by him. It should be added that many preparations have been made by a technic similar to that of the recent work of Avery and Goebel, and with many modifications, with the result that no correlation was found between the amount of distillable acid after acid hydrolysis and antigenicity for mice. The differences in these findings from those of others made a comparative study of the two products imperative. The proof of the contention of Avery and Goebel would be, of course, deacetylation followed by acetylation with a study of the three compounds. Repeated attempts to replace the acetyl group have not been successful in the sense of demonstrating an increased antigenicity in the acetylated product. Although it was found possible to acetylate the alkali-treated polysaccharide, the final compound was different in solubility and other characteristics, and yet unaltered in antigenicity. Consequently instead of acetylation after saponification, indirect methods were used: first, treatment with ammonium hydroxide, and sodium hydroxide at room temperature, and, second, hydrolysis with phosphoric acid and p-toluene sulfonic acid in the concentration used generally for saponification. Treatment with alkaline reagents of two samples prepared by different methods, one with and one without acetyl groups, did not decrease the antigenicity of the final product. This is true, despite the fact that the one from which acetic acid was originally obtained, after treatment with sodium hydroxide at room temperature, no longer yielded

acetic acid in the distillate. In other words, alkaline hydrolysis which removed acetyl groups did not decrease antigenicity. In the same way, a sample prepared by each of the two methods, one with and the other without acetyl groups, when hydrolyzed with phosphoric or p-toluene sulfonic acid, maintained the same antigenicity as the original before acid-heat treatment. The preparation by the Heidelberger method contained originally 4.5 per cent distillable acid and gave a positive test for acetic acid. After acid hydrolysis, the acid was reduced to 0.52 per cent with phosphoric acid, and 0.65 per cent with p-toluene sulfonic acid. Furthermore, the small amount of distillable acid gave a negative test for acetic acid. Yet there was no apparent decrease of antigenicity for white mice from that of the original preparations. The same is true of the sample prepared by the *calcium phosphate method, in all respects, except that the distillable* acid of the original preparation gave a negative test for acetic acid.

From the experiments given in detail here, and from others, it would appear that the acetyl group on the Type I polysaccharide has no bearing upon its antigenic activity for white mice or for men. Other changes noted in the polysaccharide by the treatments described here, such as decrease in immune precipitable nitrogen by alkaline treatment with no decrease in precipitin titer, are similar to those obtained by Heidelberger, and Avery and Goebel, and others. In addition, the variation in glucose number from some samples without demonstrable glucose to those having as high as 36 per cent, would indicate as previously stated that antigenicity is due to a definite structural configuration upon which glucose molecules are bound. But these in themselves are not essential to insure antigenicity. It is believed that immunological specificities, such as the amount of protein precipitated or precipitin titer, vary depending upon the complete polysaccharide, but that antigenicity is related to a certain organic structure so far not defined.

SUMMARY AND CONCLUSIONS

A study of the effect on antigenicity for white mice of both alkaline and acid treatments of the polysaccharide of Type I

pneumococcus prepared by both Heidelberger and calcium phosphate methods yielded the following results:

1. Ammonium hydroxide (sp. gr. 0.89) at room temperature caused but little alteration in nitrogen content, glucose number, precipitin titer, immune precipitable nitrogen, or optical rotation. Distillable acid after acid hydrolysis of one Heidelberger preparation was reduced from 7.53 to 1 per cent and of a calcium phosphate product from 2.69 to 1 per cent; yet neither distillate gave a positive spot test for acetic acid after vacuum distillation. Antigenicity of both preparations remained almost unchanged.

2. Treatment at room temperature with normal sodium hydroxide produced no change in the immunizing titer of either sample. Yet the Heidelberger material which originally showed the presence of acetic acid in the vacuum distillate, now gave a negative test. Treatment with ammonium hydroxide was without effect on antigenicity. The acid in the distillate, reduced from 4.52 to 1.72 per cent, gave a positive test for acetic acid.

3. Acid hydrolysis at 100°C. with either 5 per cent phosphoric acid or 5 per cent p-toluene sulfonic acid destroyed the acetyl groups present on the Heidelberger sample, and yet produced no change in antigenicity. Also this same treatment on a calcium phosphate preparation produced no alteration in antigenicity. From these findings, the conclusion is definite that the presence or absence of acetyl groups on Type I pneumococcus polysaccharides, prepared either by the recent Heidelberger method or the calcium phosphate method, is of no significance for its antigenicity in white mice.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN NEW YORK BRANCH

DIVISION OF LABORATORIES AND RESEARCH, ALBANY, SEPTEMBER 29, 1939

ELECTROPHORESIS METHODS FOR THE ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY IMPORTANT SUBSTANCES. *Arne Tiselius*, Professor of Biochemistry, University of Upsala, Sweden.

INDIANA BRANCH

PURDUE UNIVERSITY, WEST LAFAYETTE, INDIANA, MAY 12, 1939

AUXIN PRODUCTION BY SOIL MICROORGANISMS. *J. L. Roberts and E. Roberts*, Agricultural Experiment Station, Purdue University, West Lafayette, Ind.

One hundred and fifty species of bacteria (73 species), molds (39 species), and actinomycetes (38 species), from Indiana soil have been tested to determine their capacity to produce auxin when grown on beef-extract peptone agar and a synthetic agar containing a mineral base with asparagine, glucose, and ammonium sulphate. Streaked plates were used. All the organisms studied were aerobes. After incubation, an agar block with a volume of ten cubic millimeters was cut from beside a colony and the auxin content determined by Avena tests. No attempts were made to identify the various species studied or the auxins produced. The frequency distribution, in percentage of species tested producing auxin on beef-extract peptone agar, was as follows: gram-negative rods 79, gram-positive rods 73, cocci 84, (average of all bacteria tested 77), molds 46, and actinomycetes 66.

The average number of plant units of auxin produced in 10 cubic millimeters of agar by the various groups of microorganisms was as follows (excluding cultures which produced no auxin): gram-negative rods 20, gram-positive rods 19, cocci 21, (average of all bacteria tested 20), molds 14, and actinomycetes 18.

On the synthetic medium 23 gram-positive rods, 11 gram-negative rods, 4 cocci, 14 molds, and 23 actinomycetes were tested. Twelve gram-positive rods, seven gram-negative rods, one coccus, and two actinomycetes were found capable of synthesizing auxin from the synthetic medium. The average number of plant units of auxin produced in the 10-cubic-millimeter synthetic agar test block by the various groups of organisms was as follows (excluding cultures which produced no auxin): gram-negative rods 21, gram-positive rods 19, cocci 17, and actinomycetes 16.

COMPARISON OF METHODS USED FOR DETECTING THE ENZYME PHOSPHATASE IN DAIRY PRODUCTS.

W. H. Brown, Agricultural Experiment Station, Purdue University, West Lafayette, Ind.

A comparison of the various laboratory and short field tests devised for the detection of the presence of the phosphatase enzyme in determining the adequacy of pasteurization of dairy products has been made to determine the accuracy of each method and to determine the possible inaccuracies that may occur which are not directly due to the technique involved. The tests were applied primarily to butter to determine whether or not the cream from which the butter was made had been adequately pasteurized.

The results show that the concentration of the phosphatase enzyme is greater in cream than in milk. This indicates that, if the standard for properly pasteurized milk is to be used for cream, the temperature and time for the pasteurizing of cream must be proportionally increased.

It has been found that when butter made from properly pasteurized cream is allowed to remain without refrigeration for a few days, a positive phosphatase test will be secured.

The evidence secured indicates that the Kay and Graham, the Gilcreas and Davis and the Scharer techniques are nearly equal in the detection of inadequately pasteurized dairy products. Of the short field tests, the sensitivity of the modified test is much greater than that of the original Scharer method.

Sodium Lauryl Sulfate Broth for Coliform Detection. *E. R. Hupp*, Indianapolis Water Co., Indianapolis, Ind.

The use of 0.02% sodium lauryl sulfate in standard lactose broth for the enrichment of water samples has been suggested by Cowles (J. Am. Water Works Assoc., June 1938) to prevent the growth of spore-forming gas formers without affecting the development of coliform organisms. This is desirable in order to obtain final results of an examination earlier and to reduce laboratory work.

In one series, 118 positive presumptives were obtained in standard lactose broth, none of which confirmed. In lauryl broth, there were no positive enrichments.

One series produced 77 coliform organisms in standard lactose broth and 79 in lauryl broth. Another series produced 134 coliforms in standard and 124 in lauryl broth.

The last series produced substantially the same results obtained with brilliant green. The work is being continued.

Studies of Neutralizing Antibodies Produced by Purified Rabies Vaccine. *G. E. Hines, Jr.*, Biology Dept., Purdue University, West Lafayette, Ind.

The work presented in this thesis represents an attempt to show that purified avirulent rabies vaccine is capable of producing detectable antibodies in the serums of vaccinated animals.

To this end an *in vitro* neutralization test, utilizing white mice, was used. Equal amounts of the serum and virus dilutions were mixed, incubated for two hours and then injected intracranially into white mice. The serums to be tested were obtained at two-week intervals following the completion of the vaccination treatment. Certain variables attending this test were eliminated by appropriate experiments.

The results of the tests seemed to indicate that the purified avirulent vaccine was capable of producing viri-

cidal antibodies. The highest neutralizing action of the serums was present at four weeks, then slowly declined.

The serum from the rabbit receiving ten daily doses of ten ml. of the purified vaccine neutralized ten times the amount of virus as the serum from the rabbit receiving ten daily doses of 2 ml. of a 1% unpurified avirulent vaccine.

ANTIVIRUS PRODUCTION FOLLOWING INOCULATIONS WITH CONCENTRATED PURIFIED RABIES VACCINE. *C. A. Behrens and G. D. Canatsey*, Biology Dept., Purdue University, West Lafayette, Ind.

Serums from rabbits vaccinated subcutaneously with 0.2 and 2.0 ml. and intravenously with 2.0 ml. of concentrated purified rabies vaccine were investigated for neutralizing antibodies.

By *in vivo* protection tests, in which the antiserum was injected into white mice 48 hours prior to intracerebral injection of the virus, no neutralizing antibodies were demonstrated. By allowing the antiserum-virus reaction to occur *in vitro* at 37°C. followed by intracerebral introduction of the virus, antibodies apparently were shown to be present.

The serum from an animal receiving 2 ml. vaccine per day exhibited much greater viricidal power than that obtained from the rabbit vaccinated with 0.2 ml. The antibody production after vaccination subcutaneously with 2 ml. per day for a ten-day period apparently reached a peak four weeks after vaccination at which time 50% of the mice survived 1,000 M.L.D. of the virus. The intravenous administration of 2 ml. vaccine per day for ten days resulted in serum six weeks after vaccination which permitted 50% of the mice to survive over 200 M.L.D. as compared with 75 M.L.D. for the serum from rabbits six weeks after receiving 2 ml. vaccine per day subcutaneously for the ten-day period.

PROTECTION AGAINST RABIES. I. THE EFFECT OF FREQUENCY OF DOSAGE OF VACCINE UPON IMMUNITY. *J. M. Moss*, Lilly Research Laboratories, Indianapolis, Ind.

Many clinicians feel that, when starting of treatment is delayed, due to late diagnosis in the biting animal or in bites in close proximity to the central nervous system in exposure to rabies, it is advisable to give multiple daily injections of rabies vaccine in an attempt to stimulate a more rapid response of antibody formation. It has been the author's experience that immune response is retarded by too frequent injections of antigens.

To demonstrate that this point holds in immunization against rabies, seven groups of rabbits were immunized with a varying number of daily doses of rabies vaccine, ranging from four to one injection daily. Five groups received a total of fourteen injections of vaccine prepared by the Harris Method (attenuated vaccine). Two groups received vaccine prepared by the Simple Method (phenolized vaccine); one group receiving fourteen inoculations and the other, twenty-one inoculations.

All groups were bled at varying intervals on the same date. Serum-virus neutralization tests were made by intracerebral injection into mice. Titration against two minimum lethal doses showed little variation in response. Response to serum-virus neutralization with sixteen minimum lethal doses revealed a marked variation in response. Multiple daily doses seem to retard early response as well as the duration of immunity. The earliest immune response with the

longest duration of immunity to rabies vaccine is obtained by the administration of single daily doses of vaccine made by the Harris Method (attenuated vaccine).

A New Rapid Slide Flocculation Test for Syphilis. *L. Y. Mazzini*, Indiana State Board of Health, Indianapolis, Ind.

Mazzini described a slide flocculation test for the diagnosis of syphilis based on the use of beef heart and egg yolk extract, which has been shown to possess a high degree of specificity and sensitivity. By means of titrations the antigen is standardized to eliminate fluctuation in the sensitivity of every new lot of antigen. It employs a buffered saline solution by means of which daily fluctuations in the sensitivity of the antigen suspension are largely eliminated. The test requires a very small amount of serum for its performance. To the patient's serum, previously heated, a drop of antigen suspension is added, the slide is then rotated for four minutes and the results are read immediately under the low power of the microscope. The antigen suspension is ready for use at any given time for as long as 24 hours. Certain unsatisfactory features common to some flocculation tests have been eliminated, yielding a simplified technic.

Bacteriological Observations: from Wheat to Bread. *G. K. Ashby*, Mead Johnson & Co., Evansville, Ind.

Factors influencing bacterial deteriorations of bread were studied in bakeries and flour mills. Samples of baking ingredients were analyzed for "rope" producing organisms.

Creases in grains of wheat carry bacteria and mold spores through the mill's cleaning and tempering processes. In the mill, particularly under the first break and in the spouting after the first bolt, excellent conditions exist for bacterial and mold growth.

Conditions in bakeries approached both extremes of cleanliness. Slicing and wrapping of bread are conducive to molding and bacterial deteriorations. The most commonly overlooked source of contamination in bakeries is the pipe leading from the tank-scales to the dough mixer. Ingredients found to contribute largest numbers of "rope" bacteria were powdered milk and flour, especially whole wheat.

Methods of controlling "rope" include purchasing ingredients on bacterial specifications, sanitation, acidification, and disinfection.

Acetic acid, 2.5%, prevents growth of the vegetative cells but merely inhibits germination of the spores.

Fumigation with 3 ounces of formaldehyde per 1,000 cu. ft. kills the several "rope" species (*Bacillus*), but 2 ounces does not. Incrustations containing shortening or other greasy material definitely decrease efficiency of fumigation.

Hypochlorite spray containing 1200 p.p.m. available chlorine is effective, but 600 p.p.m. is not.

THE ENDOGENOUS RESPIRATION OF BACILLUS CEREUS

I. Changes in the Rate of Respiration with the Passage of Time

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I. INTRODUCTION

A starving bacterial cell may continue to respire for some time; *Mycobacterium tuberculosis* for example, retained an appreciable respiration, even after starving for 15 days, (Loebel, Shorr and Richardson, 1933). Stier and Stannard (1935) found that the respiration of yeast was also resistant to starvation, and proposed the term "endogenous respiration" to describe the respiration of starved cells. *Bacillus cereus* has a large endogenous respiration which persists for some time. These organisms are all gram-positive. Sevag (1933) has suggested that this character is related to high endogenous respiration, for he found that butyric acid bacilli with a low endogenous respiration failed to retain Gram's stain. Evidence is given below which shows that, in the case of *B. cereus*, the gram-positive character and the high endogenous respiration are both associated with a high content of fats in the cell.

II. EXPERIMENTAL METHODS

A strain of *B. cereus* was used, isolated from a bacon slime by the writer in 1933, and developed from a single cell culture. The strain corresponds closely in physiological characters with that preserved by the Lister Institute for type cultures (no. 2599), but it has a lower optimum temperature, (25 instead of 30°C.) and

forms very long cells in young cultures, especially in nutrient broth.

Standard cultures were produced as follows. A tube culture was made in broth every 24 hours; and when a culture had been incubated at 25°C. for 24 hours 1 ml. was removed for seeding each agar plate on which the cells for an experiment were grown. The broth had the composition:

Glucose	20 gm.	A
Distilled water	500 ml.	
NaCl	10 gm.	B
Pork extract	500 ml.	

the two solutions being sterilised separately, and the pH of B adjusted to 7.0, while hot, with 40 per cent caustic potash; the solutions were then mixed in equal proportions: the solid medium had the same composition with the addition of 2 per cent Difco Bacto-agar. Plates and tubes were incubated over-night at 25°C. before inoculation, in order to avoid temperature shock. One milliliter of the 24-hour old broth culture was seeded onto each plate, and spread by means of a bent glass rod. The cells were incubated at 25°C. until they reached the age required.

The growth from the plates was suspended in distilled water, shaken, and separated by centrifuging. This process was then repeated twice, in distilled water or buffer solution as the case might require. The thrice-washed and shaken suspension was filtered through a Schott sintered glass filter, porosity G3, into a vessel so arranged as to permit of aeration and dilution of the suspension under aseptic conditions. In this vessel the suspension was diluted with distilled water or buffer solution, to the same turbidity as a standard barium sulphate suspension, so chosen that the bacterial suspension contained about 10 mgm. dry weight of cells per ml. of suspension. A vigorous stream of air was then passed through the diluted suspension for 15 minutes, and after which 1.5 ml. of suspension was measured into each manometer; other solutions were added to these samples in por-

tions of 1.5 ml. Dry weights were determined by drying out a sample of suspension in air at 105°C. for 24 hours.

The oxygen absorption was determined in Barcroft differential manometers, shaken with a traverse of 2.5 cm. at 95 cycles per min. in a water-bath maintained electrically at 25 ± 0.05°C. To absorb carbon dioxide, 0.4 ml. of 40 per cent caustic potash was used, with 2 sq. cm. of Whatman no. 40 filter paper (Dixon 1934); with this absorbent and the rate of shaking given, experiments showed that the manometers would measure rates of oxygen uptake as high as 1,000 c.mm. per hour.

Measurements of size, and percentage of gram-positive cells, were made on samples of aqueous suspensions dried in air at room temperature. The films were stained by Jensen's modification of the Gram stain, and the organisms then measured with an eye-piece micrometer.

The fat content of the cells was estimated by a modification of the method recommended by Leathes (1925). The cells were dried at 105°C. and the dry weight determined. The residue was treated with 65 per cent caustic potash for 1 hour on the water-bath, an equal volume of alcohol added, and the whole refluxed for 1 hour. The extract was treated with 40 per cent sulphuric acid, and the fatty acids taken up in petrol ether by shaking for 1 hour and estimated by weight after evaporation of the ether and desiccation *in vacuo*. The fatty fraction determined in this way is larger than that obtained by direct extraction with petrol ether, for it contains the fatty acids from structural lipoids, which are of interest in connection with the staining reaction.

III. EXPERIMENTAL DATA

With this material, three phases of respiration could be distinguished, whatever the age to which the cells had been grown.

1. At first the rate of oxygen uptake decreased. When the period of aeration was omitted from the preparation of the suspensions, this phase was prolonged correspondingly and the initial rate of respiration was much higher. It could also be prolonged by reducing the degree of agitation during washing of the cells,

but it was not shortened by washing a greater number of times. The duration of this phase was greater the older the cells, as is shown in figure 2. It was not appreciably altered by a considerable reduction in temperature (curve *f*, fig. 2).

2. During the next phase, the changes in respiration depended very markedly on the age of the cells.

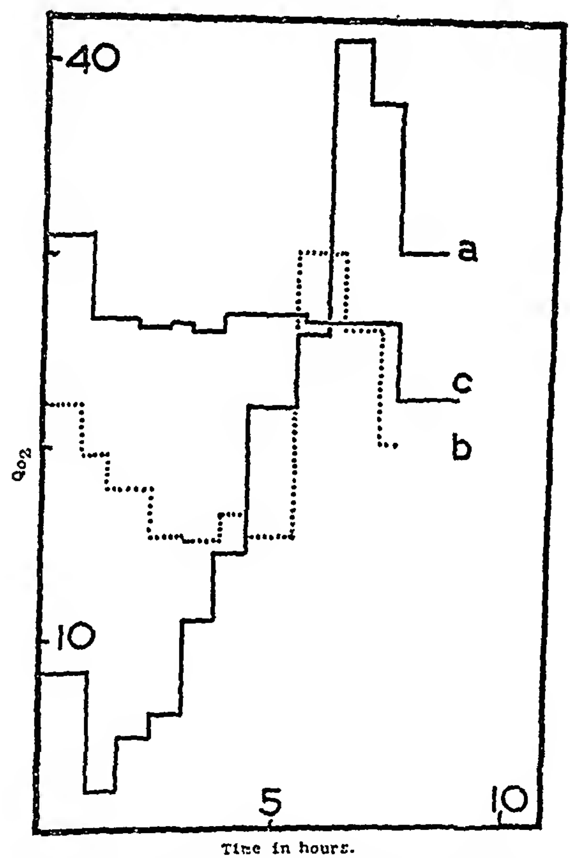

FIG. 1. THE ENDOGENOUS RESPIRATION AT 25°C. OF CELLS OF B. CEREUS GROWN TO DIFFERENT AGES: (a) 12-HOUR CULTURE, (b) 18-HOUR CULTURE, (c) 24-HOUR-CULTURE

(a) With cells from cultures less than 18 hours old at 25°C. (i.e. in the logarithmic phase of growth), the rate of consumption of oxygen rose steadily, to rather high values in the case of cells 12 hours old (curve *a*, fig. 1). The behavior of 18-hour-old cells

(curve *b*) was similar but less marked. The 12-hour cultures consisted of long cells (20–50μ) united in chains; in the course of a respiration experiment the total number of cells increased about 10 times, and the length of the cells fell to 3–5μ.

(b) With cells 24 hours old or more (i.e. from the stationary or senescent phases of growth), the total number of cells did not change appreciably, nor did the rate of uptake of oxygen for a

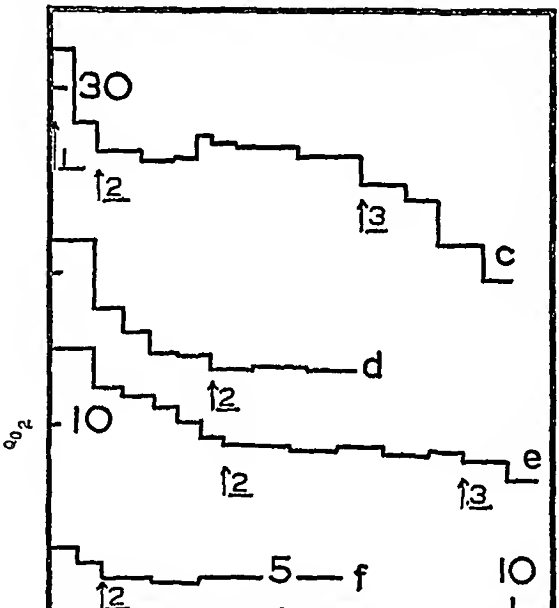

FIG. 2. THE ENDOGENOUS RESPIRATION OF CELLS OF B. CEREUS GROWN TO DIFFERENT AGES

(c) cells 24 hours old, measured at 25°C., (d) cells 48 hours old, measured at 25°C., (e) cells 72 hours old, measured at 25°C., (f) cells 24 hours old, measured at 1°C. The phases of respiration are indicated by arrows.

period of several hours. The rate of endogenous respiration during the period of constancy was lower the older the cells (curves *c–e*, fig. 2). At the end of this phase of respiration, the viable count was always less than 1 per cent of the total number of bacteria.

3. In the final phase, the rate of absorption of oxygen declined following an exponential course with time for at least 10 hours

(curve B, fig. 3). Precautions were taken to ensure that contaminating organisms were absent, that oxygen was adequate, and that carbon dioxide did not accumulate in the manometers. This decline of respiration was common to cells of all ages.

The presence of salts did not interfere with the appearance of these phases of respiration; low concentrations caused a slight

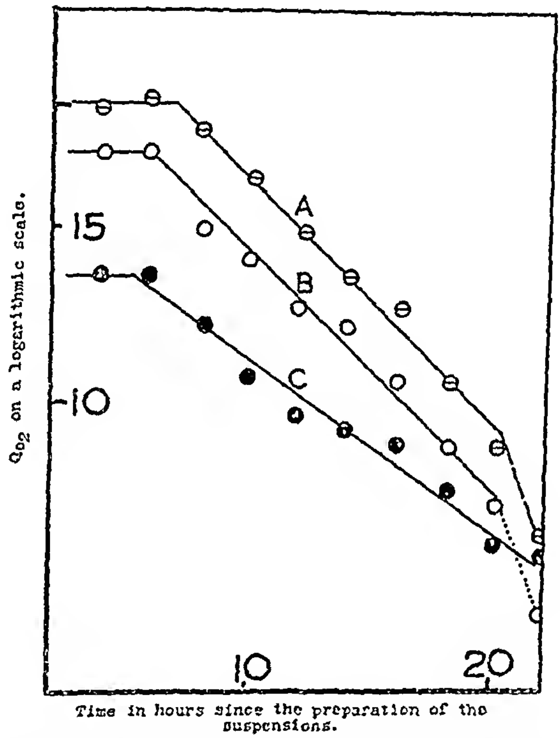

FIG. 3. THE DECLINE, AT 25°C, IN THE RATE OF UPTAKE OF OXYGEN BY CELLS OF B. CEREUS SUSPENDED IN (A) 0.01M NaCl (B) DISTILLED WATER (C) 0.6M NaCl

increase and higher concentrations a decrease in the rates of respiration. It was noted, in particular, that with mature cultures the level of respiration during the second, constant, phase could be changed by sodium chloride, without altering the time at which the respiration began to decay.

To test the possibility that some inhibitor might be produced

during respiration, a sample of cells was aerated in distilled water for 20 hours at 25°C., and the suspending fluid centrifuged off. This might be supposed to contain the inhibitor. Samples of a freshly-prepared suspension were then diluted with an equal volume of distilled water, or the solution centrifuged from the starved cells. It was found that the respiration of the suspension containing "inhibitor" (in half the concentration present in the starved suspension) was about 5 per cent less than that of the normal suspension. However, the pH of the normal suspension was found to be 6.5 at the end of this experiment, and that of the treated suspension 7.0. This difference accounts for the difference between the rates of respiration, so that the "inhibitor" was a substance making the suspensions more alkaline. After 20 hours starvation, the pH of a normal suspension was 7.5, and this was not alkaline enough to account for the low respiration observed.

The lost respiration could not be restored by feeding with glucose. With starving 24-hour-old cells in the phase of constant respiration, the Q_{O_2} (= c.mm. oxygen per hour per mgm. dry weight) was raised roughly from 20 to 40 by the addition of glucose. When the Q_{O_2} of endogenous respiration had fallen to 5, addition of glucose raised the Q_{O_2} only to 7.5. Thus the increase in respiration due to glucose was only $\frac{1}{8}$ of that attained earlier, a fall greater in proportion than that of endogenous respiration. The increase in rate of respiration was independent of glucose concentration within the range 0.005–0.5 M; a similar state of affairs has been reported to occur with *Sarcina lutea* (Gerard and Falk, 1931).

It was found that the respiratory quotient $\left(\text{R.Q.} = \frac{\text{vols. } CO_2}{\text{vols. } O_2}\right)$ remained low throughout. With cells 24 hours old, respiring at 25°C. in phosphate buffer at pH 6, it had an initial value of 0.5; during the second phase of respiration it remained roughly 0.6, and rose gradually to 0.7 after 15 hours. Analysis of cells of *B. cereus* showed that the low R.Q. was accompanied by a disappearance of fat from the cells. Starvation for 23 hours at 25°C.

reduced the fat-content from 4.2 per cent to 1.0 per cent of the dry weight. The loss of fat was accompanied by a diminution in the volume of the cells, and by a loss of the ability to retain Gram's stain. These changes are shown in table 1.

B. cereus is normally gram-positive, and even in old cultures all the cells retain stain. Starving of 24-hour-old cells for only 5 hours was sufficient to make half of the cells gram-negative, and even those which continued to retain the stain held it only in small granules, apparently on the margin of the cells. The microscopic appearance of the cells at different stages of starvation was in keeping with the supposition that the diminished volume of the cells resulted from a loss of their outer layers.

TABLE 1

Changes in fat content, cell-volume, and Gram reaction, during starvation of 24-hour-old cells of B. cereus at 25°C.

HOURS OF ENDOGENOUS RESPIRATION AT 25°C.	FATTY ACIDS AS PERCENTAGE OF DRY WEIGHT	AVERAGE VOLUME OF A CELL (μ^3)	PERCENTAGE OF CELLS RETAINING ANY STAIN
0	4.2	5.0	100
2		2.6	62
4		2.4	58
6	2.4		43
6		2.6	46
8		1.7	25
12	1.8		25
13		1.7	34
15		1.0	16
23	1.0	1.4	8

The average volume and percentage of gram-positive cells were determined for 100 cells by two independent observers.

IV. DISCUSSION

An initial phase of falling respiration has been observed to occur with *Sarcina lutea* by Gerard and Falk (1931). They attributed it to the accumulation of an oxygen debt from asphyxia during the washing of the cells, the debt being met gradually as the cells became saturated with oxygen. Shoup (1929) has shown clearly that such an oxygen debt is accumulated during anaerobiosis. The relations to washing and aeration support the view

that this took place with *B. cereus.* The unduly large uptake of oxygen would account for the low R.Q. If the process were controlled by the diffusion of oxygen into the cells, one would expect it to be largely independent of temperature, as was the case. Moreover, the increased duration when older cells were used may be an expression of their lower permeability.

With actively-dividing cells from young cultures division appears to have been continued on suspension in distilled water, without any corresponding increase in cell material. The high rate of respiration associated with this process presents analogies to the high rates of metabolism observed when bacterial cultures emerge from the lag phase of growth by the cleavage of large cells into smaller cells of normal size. (Martin, 1932; Bayne-Jones and Adolf, 1932; Walker *et al.* 1934).

With mature cells there seemed to be a period of equilibrium with the menstruum, for no division occurred and the respiration remained constant. This has been observed in *B. subtilis* (Callow, 1924) and in yeast (Stier and Stannard, 1935). The equilibrium was disturbed by some cause which brings about an exponential decline with time in the rate of respiration. This might be attributed to (i) death of the cells, (ii) the production of some inhibitor during respiration at the end of the period of equilibrium, (iii) the exhaustion of a food-supply once it is reduced below a certain critical level or (iv) the decay of the enzyme systems of the cells according to a monomolecular law.

(i) It has been shown, e.g. by Rahn (1929), that under certain conditions the viable fraction of a population of cells may remain constant for some time and then decline, the decline being roughly exponential with time only in its later stages. Although the general behaviour of endogenous respiration was similar, the decline was exponential throughout. Moreover, the interpretation in the case of viability depends on the fact that this is an "all or none" property: there is no evidence that the respiration of a single cell ceases abruptly in the same way. The changes in endogenous respiration were not directly related to the viability of the cells, since it was reduced to one-hundredth without any corresponding change in respiration. This probably took place

principally during the first phase of respiration, for Winslow and Brooke (1926) found the viability of washed cells of *B. cereus* to be reduced by 99 per cent after exposure to distilled water for 1 hour. Dieckman (1934) quotes many instances in which, during a period of constant respiration, the number of viable cells fell to an insignificant proportion of the whole.

(ii) An experiment has already been described which showed that specifically inhibiting substances were not responsible for the low respiration of starved suspensions. Their alkalinity was probably due to the production of ammonia and carbon dioxide (compare Shaughnessy and Winslow, 1927), the latter being removed by the potash in the manometers while the ammonia accumulated in the suspensions.

(iii) Working with yeast cells more than 2 days old, Stier and Stannard (1935) observed phases of constant respiration and of exponential decay with time. They believed that this represented the exhaustion of the glycogen reserve, and that decay set in directly its concentration fell below a critical value. This conclusion was based on 3 observations. The first two of these were that glycogen disappeared and that the R.Q. remained 1 throughout endogenous respiration; these facts proved that a carbohydrate was the substrate of respiration. The third was that sugar fed to starved cells caused a large increase in respiration, the R.Q. remaining 1; this showed that the enzyme system attacking carbohydrate was intact after starving. This hypothesis does not apply to *B. cereus*, for two reasons. Firstly, when the rate of utilisation of the food reserve was diminished by salt, the period of constant respiration was not prolonged (curve *C*, fig. 3). Secondly sugar-feeding indicated that the respiratory enzymes were destroyed during starvation, those oxidising glucose even more than those responsible for endogenous respiration.

(iv) The data of Martin (1932) demonstrated that there is a close relation between the surface area of a bacterial cell and the rate at which it utilises oxygen; this would occur if the amount of the respiratory enzymes were related to the area of the superficial layers of the cell. The simultaneous diminution in the size of cells of *B. cereus* and in their ability to retain Gram's stain sug-

gests that it was these outer layers that were respired away; two observations indicated that this was fatty material. The low R.Q. was associated with a fall in the total fatty acids of the cell; Stephenson and Whetham (1922, 1923) found that low values of the R.Q. were associated in *Mycobacterium phlei* with the oxidation of fat in the cell. Secondly, Churchman (1929) and Burke and Barnes (1929) have shown that the power of retaining Gram's stain lies in the outer sheath of bacteria, which agrees with the microscopic appearance of starved cells of *B. cereus*, while Jobling and Peterson (1914) and Tamura (1914) have shown that the gram-positive character is associated with fat in the cell, an association confirmed for *B. cereus*.

If it be supposed that during the third phase of endogenous respiration (1) the outer sheath and respiratory enzymes were destroyed at a rate proportional to the rate of respiration, and (2) that the rate of respiration was proportional to the amount of enzymes remaining, we have—

$$-\frac{dE}{dt} = K \cdot E_t$$

(where E_t is the amount of enzyme remaining at any instant) and thus

$$\ln E_t = C - K \cdot t$$

(C and K constant) whence

$$\log R_t = \log R_o - K' \cdot t$$

(t being measured from the instant at which the decline of respiration begins)

This is the relation represented by the falling straight lines of figure 3, the slopes of the lines representing values of K'. Thus the exponential decline of endogenous respiration may be related to the destruction by respiration of an outer, fatty, gram-positive sheath of the cell, containing the respiratory enzymes.

Sevag (1933), from a study of butyric acid bacilli, came to a similar conclusion. These bacilli were gram-positive in young, and gram-negative in old cultures. Data were obtained which are shown in table 2.

The loss in the ability to retain Gram's stain was concomitant with a diminished endogenous respiration, and a corresponding loss of the ability to utilise added sugar. In these cases the changes in respiration were almost certainly due, in the main, to the differing ages of the cells, since there are similar but smaller differences in the respiratory activity of cells of *B. cereus* at different ages without any change in the gram-reaction. However, Sevag also stated that the same changes could be brought about by starving butyric acid bacilli, and that during starvation the cells became smaller. These statements, which are amply confirmed by the data presented above for *B. cereus*, led Sevag to the same conclusion: that the respiratory enzymes, and the loci

TABLE 2

Intensity of respiration and Gram reaction in butyric-acid bacilli

(Calculated from the data of Sevag, 1933)

AGE OF THE PARENT CULTURE	PERCENTAGE OF GRAM-POSITIVE CELLS	Q_{O_2} IN:	
		Buffer	Buffer + glucose
hours			
44	6	0.5	2.0
24	55	8.5	21.5
14	92	20.5	62.5

retaining Gram's stain, are situated in the ectoplasm of the cells, and are respired away during starvation.

V. SUMMARY

When cells of *Bacillus cereus*, grown on nutrient agar at 25°C. for less than 24 hours, are washed and suspended in distilled water or buffer solution cell-division continues, and gives rise to an unusually high rate of respiration.

If the cells are 24 hours old, or older, no cell division occurs, and the endogenous respiration passes through three phases.

The first phase usually lasts less than two hours. Its duration is greater with older cells, but is not much affected by temperature. The large uptake of oxygen is attributed to the satisfaction of an oxygen debt arising from asphyxia during washing.

The second phase lasts about five hours at 25°C. and during this time the rate of respiration remains constant. The magnitude of this constant rate decreases with increasing age of the cells.

During the third phase, the rate of respiration declines exponentially with time. The time which elapses before this phase of respiration begins is largely independent of the rate of respiration with cells of any given age, which suggests that the decline in respiration is not brought about by the failure of a food supply.

During endogenous respiration the cells diminish in size and lose their fatty materials and their gram-positive reaction. The low R.Q. (0.7) indicates that the fat is probably destroyed by respiration, and this apparently leads to the loss of the outer gram-positive sheath of the cells. The exponential decline of the respiration accords with the view that the respiratory enzymes are destroyed during this phase, and thus associates the respiratory enzymes with the gram-positive sheath of the cell.

The addition of sodium chloride does not destroy the three phases of endogenous respiration. Its effect on respiration may therefore be measured with convenience, by comparing the constant rates of respiration during the second phase, in the presence and absence of salt.

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THE ENDOGENOUS RESPIRATION OF BACILLUS CEREUS

II. The Effect of Salts on the Rate of Absorption of Oxygen

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I. INTRODUCTION

This paper deals with the action of salts on respiration in the absence of substrate—the endogenous respiration of Stier and Stannard (1935). The use of substrates which permit cell division has been avoided because salts influence cell multiplication, creating difficulties in interpreting the results in terms of the effect of the salts upon respiration. Moreover, measurements of gaseous exchange in the presence of substrate may give a misleading picture of the respiratory activity of a bacterial suspension owing to the existence of concurrent anabolic reactions, even where the number of cells does not change, as in the case of washed cells (Giesberger, 1936; and Barker, 1936). The effects of the components of the buffer mixture were first assessed, since they were expected to be similar to those of other salts. Thus, the investigation was begun by studying the effect of salts in unbuffered solutions without substrate, and the data so obtained were applied to the interpretation of experiments in which the effect of salts was studied in buffered solutions.

II. EXPERIMENTAL PROCEDURE

The comparison of the effects caused by a variety of salts necessitated the preparation of a series of cultures of the same physiological age, washed so as to remove external nutrients. Suspensions of these cells were mixed with salt solutions, and the consumption of oxygen was then followed in manometers.

The conditions under which the cells were grown have been given elsewhere (Ingram, 1939). It was found that cells 24 hours old at 25°C. had a large endogenous respiration, maintained at a constant level for several hours after the preparation of a suspension; and as it was desirable to have some constant value, to which the rates of respiration in salt solutions might be referred, cultures were incubated for 24 hours in these experiments. The growth was washed in the centrifuge, once in distilled water and then twice in distilled water or in buffer solution as the case required. The washed suspensions were shaken and filtered, and diluted to a fixed turbidity, corresponding to 10 mgm. dry weight of cells per milliliter, checked by determining the dry weight of a sample of the suspension after drying at 105°C. for 24 hours. The diluted suspension was then aerated for 15 minutes, after which portions of 1.5 ml. were measured into the flasks of Barcroft manometers and 1.5 ml. of salt solution was added to each sample. Each manometer flask thus contained a given number of cells in a given volume of salt solution. The rates at which the treated samples took up oxygen were then measured at 25°C. (Ingram, 1939).

Solutions were made in distilled water, containing about 30 parts per million of copper. The salts used were "Analar" reagents, except for the chlorides of lithium and cerium which were of "technical" purity.

III. EXPERIMENTAL DATA

Preliminary experiments

At the outset, it was necessary to decide whether the action of salts depended on the amount of salt present in relation to the number of bacteria, or on the concentration of the salt. Normal experiments would not have distinguished between these two possibilities, for equal numbers of cells were always added to the same volume of each salt solution. Special experiments were therefore carried out, with suspensions of cell-content 2.5,5 and 10 mgm. dry weight per milliliter. It was found that the rate of oxygen uptake by each of these three suspensions was reduced in the same proportion after the addition of 0.6 M sodium chlo-

ride. Thus it was presumed that the rate of endogenous respiration in a salt solution was determined solely by the concentration of salt present, and that it was legitimate to array rates of respiration against molar concentrations of salts in the suspensions.

All the data were handled in this way. The rates of oxygen absorption were steady rates attained about 100 minutes after the addition of the salts to the freshly-prepared bacterial suspension; they are expressed as percentages of those in the absence of added salts, that is, of the rates in distilled water or in buffer solution. Measurements of the rate of respiration in a salt solution were reproduced to within 5 per cent in duplicate experiments, when calculated as percentages of the rate in the absence of salt. The variation was due to differences in the susceptibility of the bacteria to salt solutions, rather than to errors in the measurement of the respiration.

The action of salts on respiration was found to be reversible. For example, addition of 1.0 M sodium sulphate to an aqueous suspension reduced the rate of respiration to one-tenth of the initial value; yet when the cells were centrifuged out after one hour in sodium sulphate and washed and re-suspended in distilled water, the rate of respiration returned to its initial value within 20 minutes. It appears that salts do no permanent injury to the respiratory system.

Data obtained with unbuffered suspensions

All the salts investigated behaved in the same way; in low concentrations they increased the rate of uptake of oxygen by *Bacillus cereus* and in high concentrations they reduced it.

The rates of endogenous respiration in the presence of small amounts of a number of salts are given in table 1. The range of concentration, over which stimulation occurred, was lower, the greater the valency of the cation. The concentrations of the chlorides of 1-, 2- and 3-valent cations which maintained the rate of respiration at the same value as in distilled water were roughly 0.1 M, 0.01 M, and 0.0001 M respectively. On the other hand, sodium chloride, sulphate, and citrate, exerted a stimulating action at concentrations less than 0.2, 0.2, and 0.05 M respec-

tively; these concentrations are so much more nearly alike that it may be supposed that the nature of the anion was of minor importance compared with that of the cation.

In table 1 are included a series of measurements made upon cells suspended in lactose solutions. These were made in order to determine the extent to which the osmotic pressure of the solution might affect the respiration of cells suspended in it. This sugar is not attacked by the strain of *B. cereus* used in these experiments. The rate of respiration was shown to be practically

TABLE 1

The increased rate of respiration in the presence of low concentrations of salts

SALT	NUMBER OF EXPERIMENTS	MOLARITY OF SALT PRESENT								
		0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2
Lactose	2			102	105	98	98	99		92
NaCl	5			120			110	104		103
KCl	3						106			106
LiCl	3					128	116	115	119	83
NH_4Cl	2					104		113		94
Na_2SO_4	2					109		107		105
$Na_3C_6H_5O_7$	4		119			150		104	62	
$CaCl_2$	4	119		112	108		97	92		80
$MgCl_2$	1							101		
$CeCl_3$	2	Concentration		*0.00005*	*0.00015*	*0.0005*		*0.006*	*0.01*	
		Respiration		104	102	81		63	43	
		pH		5.66	5.58	5.30		5.06	4.60	

independent of lactose concentration up to 0.2 M, which indicates that salts do not increase respiration by changing osmotic pressure.

The data relating to the chloride of a trivalent metal, cerium chloride, are included in table 1. They are not directly comparable with those for other salts by virtue of the acidity developed in the solutions. (It would have been possible to overcome this condition by the addition of alkali along with complex ions to keep the trivalent hydroxide in solution, but the interpretation

of the data appertaining to such solutions would have been difficult.) It would appear that the chloride of the trivalent metal was a more potent inhibitor than those of mono- or di-valent metals, but the concentrations of hydrogen-ions in the solutions were such that it was impossible to ascribe the decrease in respiration to the salt alone.

A study of the inhibition of respiration under the influence of more concentrated salt solutions showed that, above a certain limit of concentration, the rates of absorption of oxygen frequently approximated to those given by the equation

$$\log r = P - Q.c. \qquad (1)$$

(where r is the rate of uptake of oxygen, c the concentration of salt in the suspension, and P and Q are constants for a given salt). By plotting log r against c a linear relation was obtained, whether r was measured in absolute or in relative units, for multiplication of the value of r merely changed the value of P. Thus, in the figures, the percentage rates of respiration (referred to a suspension without salt) are plotted on a logarithmic ordinate, against the salt concentration on a linear abscissa. (With this arrangement, the value of Q is given by the slope of the line log r/c, and the value of P by the intercept of the line on the ordinate axis. Q is a measure of the proportion in which respiration is decreased by a given increase in salt concentration. P corresponds to the logarithm of a fictitious rate of respiration in the absence of salt, which is usually higher than that actually observed, since the rate of respiration passes through a maximum value at low salt concentrations; this portion of the respiration/concentration relation is not shown in figures 1 and 2, which portray only that range of salt concentrations over which equation (1) has been found to hold good.)

Figure 1 shows how closely the respiration obeyed equation (1) within the appropriate range of concentration. Roughly speaking, equation (1) was valid directly there was any considerable inhibition of respiration. Figure 1 shows, in addition, that the value of Q increased with the cations of alkali metals in the order K^+, Na^+, Li^+, the values being 0.32, 0.68 and 0.90 respec-

tively. Anomalous data were obtained with ammonium chloride. At concentrations less than 0.4 M the inhibition was about the same as that of potassium chloride, but it increased very rapidly at concentrations approaching 1.0 M. This was probably caused by the increased acidity (pH = 5.3 in 1.0 M solution). The action of salts of divalent cations is demonstrated by magnesium and calcium chlorides. These salts were more powerful

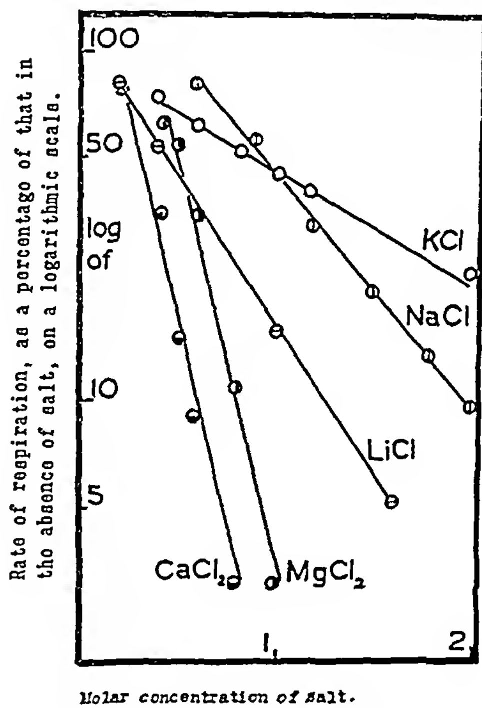

FIG. 1. THE INHIBITION OF RESPIRATION BY CONCENTRATED SOLUTIONS OF METALLIC CHLORIDES

inhibitors of respiration than those of the alkali-metals. Magnesium chloride was found to be slightly less potent than calcium chloride, although the associated values of Q and hence the slopes of the lines in figure 2 were about the same (2.24 and 2.37 respectively). The way in which the inhibition of respiration varied among sodium salts containing different anions may be seen in figure 2. The degree of inhibition caused by a given

concentration of a sodium salt increased with the valency of the anion. The values of Q for the chloride, sulphate, and citrate, calculated from figure 2, are 0.68, 1.27, and 1.58 respectively.

Mixtures of sodium and calcium chlorides were observed to give rise to effects similar to those caused by these salts taken separately. Three combinations of these salts are possible, where (a) both are present in stimulating concentrations, (b) one

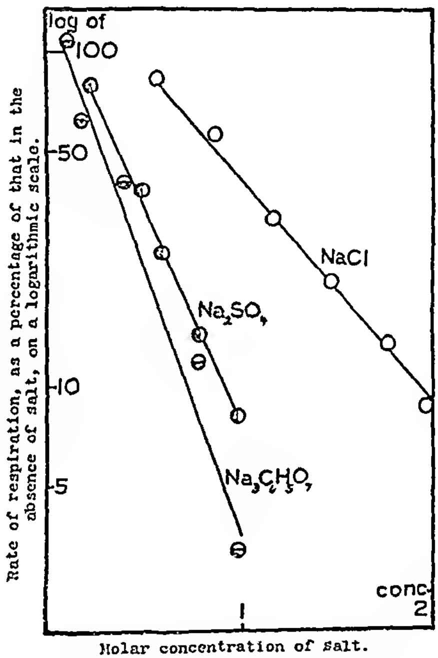

FIG. 2. THE RATE OF RESPIRATION IN CONCENTRATED SOLUTIONS OF DIFFERENT SODIUM SALTS

is present in stimulating, the other in inhibitory concentration, and (c) where both are present in inhibitory concentrations; these three possibilities are represented under corresponding letters in table 2. In dilute mixtures (a) there was slightly greater respiration than in either of the components alone, the greatest respiration occurring in a solution with a Na^{+}/Ca^{++} ratio of 10:1. Wilson (1922) has shown, correspondingly, that Ringer's solution lowers the viability of bacteria less than

physiological saline. A similar state of affairs was found in case (b) where the weakly inhibitory action 0.02 M $CaCl_2$ was converted into a considerable stimulation of respiration by the presence of nine times its concentration (0.18 M) of NaCl. But in mixtures with more calcium, the rate of respiration was lower than in either salt alone, and this was even more striking when both salts were present in inhibitory concentration.

TABLE 2

The rates of respiration in unbuffered mixtures of sodium and calcium chlorides

TOTAL MOLARITY	NaCl	$CaCl_2$	$\frac{Na+}{Ca++}$	RESPIRATION	EQUIV. CONC. NaCl*	CORRESP. TO EQUIV. CONC. NaCl†
	M	*M*				
(a) 0.03	0.03	0.0005	60:1	127	0.03	108
	0.03	0.001	30:1	137	0.033	107
	0.03	0.003	10:1	139	0.04	106
(b) 0.02	0.18	0.02	9:1	117	0.24	103
	0.16	0.04	4:1	87	0.28	90
	0.1	0.1	1:1	14	0.40	70
(c) 1.0	0.9	0.1	9:1	30	1.2	30
	0.8	0.2	4:1	10	1.4	25
	0.5	0.5	1:1	<1	2.0	8

*† The figures in these columns represent (*) a concentration = (the concentration of NaCl present + 3 times that of the $CaCl_2$ present) and (†) the respiration corresponding to this concentration of sodium chloride.

Data obtained in buffered solutions

The concentrations of hydrogen ions were not the same in suspensions containing different neutral salts because different salts affect the buffering action of a bacterial suspension in different ways (Shaughnessy and Falk, 1924). Divalent cations prevented the production of alkaline substances more than monovalent cations, giving more acid suspensions. To compare the effects of salts on respiration at a more nearly constant pH, experiments were carried out in buffered solutions.

It was found that a mixture of disodium hydrogen and potassium dihydrogen phosphates was satisfactory, if the total con-

centration was small. The cations present behaved as in other salts. The pH of an aqueous suspension of *B. cereus* remained at about 6.15 during the period of constant respiration, and the rate of respiration in phosphate buffer at this pH was some 3 per cent greater than that in distilled water. The total concentration of $Na+$ and $K+$ in this buffer was about 0.03 M, so that the increase of respiration corresponded with that obtained with other salts (cf. table 1). In buffer solution of pH 6.0, containing 0.028 M $K+$ and 0.01 M $Na+$ (Sørensen, 1909) the rate of respiration was 3 per cent lower than in buffer of pH 6.15; this drop in respiration is consequent upon the small movement in the acid direction away from the pH of optimum respiration (about 6.5). Thus, the rate of respiration in buffer at pH 6.0 was the same as that in distilled water, and the experiments were carried out at pH 6.0 so as to make the data in buffered solutions numerically comparable with those for unbuffered solutions.

The data derived from this series of experiments are set out in table 3, which shows that in buffered suspensions the general effect of salts was the same as in unbuffered suspension, with slight changes in the magnitude of the induced phenomena. Thus, comparison of the data given in tables 1 and 3 for sodium chloride, and sodium sulphate shows that in the case of each salt, low concentrations brought about a greater stimulation of respiration in the presence of the buffer than in the unbuffered suspensions. The stimulating action of low concentrations of a salt thus appears to have been increased by greater acidity.

Two salts were used which were found to cause a marked inhibition of respiration, even at low concentrations; these were sodium nitrite and potassium cyanide. The data are given in table 3. Those relating to the nitrite solutions show that there was a stimulation of respiration in the most dilute solutions, of the same order as that observed with other salts of alkali metals, the toxic effects of the anion becoming apparent only at higher concentrations. The data relating to potassium cyanide are only approximate, because hydrolysis of this salt was accelerated by reaction between potash and the hydrogen cyanide from the gas phase in the manometer flasks; this gave rates of respiration

which increased with time, as the inhibition was reversible. The data are calculated from the initial rates of respiration, and are more reliable the more concentrated the solution to which they refer:

The rates of uptake of oxygen, in buffered suspensions containing considerable concentrations of sodium chloride, did not yield a straight line when log *r* was plotted against *c*. The respiration was more strongly inhibited at high salt concentrations than in the unbuffered solutions (cf. fig. 1 and table 3). This was probably because the addition of salts to a phosphate buffer causes the pH to change in the acid direction (Green, 1933), and change of pH to values more acid than 6 contributes to the lowering of respiration with *B. cereus*.

TABLE 3

The rate of respiration in salt solutions buffered at about pH 6.0, in 0.033 M phosphate

SALT	NUMBER OF EXPERIMENTS	MOLARITY OF SALT PRESENT											
		0.0005	0.002	0.005	0.01	0.02	0.05	0.067	0.2	0.5	0.8	1.0	2.0
NaCl	5		117	126	122	112	111		105		86	50	3
KCl	2			114	104	104	98		94		67	45	
NaCl + KCl (1:1)	2			124	115	109	107		100		80	47	
$NaNO_3$	2			119	114	106		103	79		35	3	
KNO_3	2			115	109	108		103	96		57	32	
$NaNO_3$ + KNO_3 (1:1)	2			119	114	107		96	95		38	7	
Na_2SO_4	2	128	131	130		116	106		105	92			
$MgSO_4$	2	128	124	115		105	93		83	79			
$MgCl_2$	2	117	120	113		101	97		85				
$NaNO_2$	2	124		97	64	3	0						
KCN	2	75	39	32		20	20						

Observations made in buffered solutions are a little difficult to interpret, as the buffer salts might be expected to exert antagonistic influences on other ions added to the solutions. Thus, the relatively greater increase in respiration caused by sodium or

potassium chloride when in buffered solution might have been attributed to a disturbance of the Na+/K+ ratio following the addition of Na+ or K+; but as the two chlorides behaved similarly, this did not seem likely. This was borne out by the further observation that addition of a mixture of sodium and potassium chlorides caused alterations in the rate of respiration, intermediate between those caused by either salt added separately. Mixtures of sodium and potassium nitrates behaved in the same way, (with the exception of one anomalous experiment in a mixture of 0.067 M total concentration, giving a low average), confirming the observations in solutions of the mixed chlorides (cf. table 3).

IV. DISCUSSION

The data presented in section III justify the conclusion that all salts act similarly, the differences lying in the concentration of each salt needed to produce a given effect. Winslow and Dolloff (1928) reached the same conclusion from studies of the viability of *E. coli.* It is therefore unlikely that the increased respiration in the dilute salt solutions resulted from the replacement of ions leached from the cells during washing. The two salts found to influence the respiration of *B. cereus* in an unusual manner are both known to stand in special relation to cellular respiration. Sodium nitrite, especially in acid solutions, causes a reduction in the activity of dehydrogenase systems (Quastel and Wooldridge 1927). This has been interpreted as the result of a reaction between nitrous acid and the amino-groups of the dehydrogenases (Myrbäck, 1926). Potassium cyanide inhibits the cytochrome system (Keilin, 1929) and the present writer found on spectroscopic examination that *B. cereus* contains cytochrome. About 20 per cent of the respiration in *B. cereus* appears to be cyanide-stable.

Cerium chloride in rather low concentrations was found to inhibit respiration, but in this case the inhibition may probably be attributed in part to the high acidity of the solution as well as to the polyvalent nature of the cerium ion, except in the most dilute solutions. The effects of dilute solutions of sodium, calcium and cerium chlorides in increasing the endogenous

respiration of *B. cereus* were the same at concentrations roughly in the ratio of 1000:100:1. Buffering of the suspensions at a slightly more acid pH made no appreciable difference; the stimulating action of the salts was perhaps increased a little. Further, it was shown that the stimulating effects of different sodium salts were much the same, so that the anion of the salt can be little concerned in causing increased respiration.

Nicolai (1926) observed an increase in the rate of uptake of oxygen by different bacterial species in buffered glucose solutions in the presence of salts. He interpreted his data in terms of osmotic pressures of the salt solutions, and decided that for salts of alkali metals the optimum respiration occurred at an osmotic pressure 1.5 times that of blood with *Escherichia coli*, and 1.0 times that of blood with staphylococci. He observed, however, that calcium and magnesium chlorides in equivalent concentrations depressed the rate of respiration of the staphylococci, and this leads one to doubt the validity of the interpretation which he placed upon his results. The experiments in which lactose was added to suspensions of *B. cereus* showed that the endogenous respiration must be independent of osmotic pressures up to 10 atmospheres, and that the effects of dilute salt solutions cannot be attributed to osmotic causes.

Fabian and Winslow (1927), after an examination of the viability of *E. coli* in the presence of different sodium salts, concluded that the changes in viability were accounted for by differences in pH and in Na+ concentration between the different solutions, the action of the anions of the salts being negligible. This conclusion is similar to that reached from a study of the respiration of *B. cereus*. The absence of any effect due to the anions, and the ratios 1000:100:1 between equivalent concentrations of 1, 2 and 3-valent cations, recall the similar relations found when salts precipitate electro-negative colloids. Winslow, Falk and Caulfield (1923) have shown that the cell of *B. cereus* is electro-negative at any pH between 3 and 10, but this does not prove that the proteins concerned in respiration are electro-negative. It is hard to imagine how flocculation of a protein could be directly related to increased rate of respiration; never-

theless, one must suppose that the same property of the cations is concerned in the two processes.

The inhibitory powers of metallic cations, measured by the values of Q in the equation

$$\log r = P - Q.c. \qquad (1)$$

differed in accordance with a Hofmeister series, the anomalous behaviour of NH_4+ being explained by the acidity of its solutions. The ions $Na+$ and $Ca++$ or $Mg++$ were equally potent in concentrations roughly as 3:1; it is not possible to form a reliable estimate of the inhibition caused by trivalent cations. The inhibitory powers of anions are also difficult to decide. For example, at a given molarity of salt the concentration of sodium-ion is approximately three times in sodium citrate, and in sodium sulphate twice that in sodium chloride solution; thus solutions of these salts may be compared on the basis of equal content of $Na+$ by dividing the appropriate values of Q ($\log r/c$) by 1, 2 and 3. This gives Q the values of 0.68, 0.63 and 0.53 for the chloride, sulphate, and citrate, the differences between these values being due to the different anions. Thus, substitution of chloride by sulphate, or of sulphate by citrate, results in a reduction of the value of Q, despite the fact that under the conditions of comparison a given concentration of chloride would be replaced by only half that concentration of sulphate, or one-third that concentration of citrate. Increasing the valency of the anion of a salt thus reduces the potency of the cation as an inhibitor, and in this sense anions and cations may be termed antagonistic. At the same time, a change in the valency of the cation results in an increase in the value of Q greater than the decrease resulting from a corresponding difference in the valencies of a pair of anions, so that the inhibition due to salt is determined mainly by the cation.

The data obtained by Brooks (1919, 1920), measuring the inhibition of respiration by *Bacillus subtilis* in concentrated salt solutions, may be shown to accord with an equation similar to (1). The relative potencies of $Na+$ and $Ca++$ or $Mg++$ were about 1:3 in her experiments, and the effects of $K+$ were

less than those of Na+. Her data from solutions containing La+++ agree closely with those for *B. cereus* in solutions containing Ce+++, and were presumably equally influenced by changes in pH. The figures obtained by Rubinstein (1932) with *Sarcina lutea* may also be arranged in a similar manner, although with *S. lutea* the changes brought about by salt require much higher concentrations than with the bacilli. The rates of oxygen uptake by *E. coli,* measured by Nicolai (1926), conformed to the requirements of equation (1) in solutions of moderate concentration. In the most concentrated solutions the rates of respiration were lower than those which would be in agreement with equation (1); but these experiment were carried out in the presence of phosphate buffer, and the enhanced action of high concentrations of the salts is similar to that observed in buffered suspensions of *B. cereus,* but greater in degree as the respiration of *E. coli* is more sensitive to acidity than that of *B. cereus.* It would seem that equation (1) may be widely applicable. A similar relation describes the changes in the solubility of proteins in concentrated solutions of salts. The equation is

$$\log S = b - K.u. \qquad (2)$$

where S is the solubility of the protein, u the ionic strength of the salt solution ($u = \frac{1}{2} \Sigma mv^2$ for all the ions present where m and v represent the molarity and valeney of a single ion), and b and K are constants for a given salt and a given protein (Cohn 1932).

Antagonistic action between pairs of cations was negligible. Antagonism between Na+ and Ca++ implies the *reduction* of the inhibitory action of their solutions by the presence of the other ion: in the experiments of table 2 the inhibitory action of the ions was *increased* when they were in combination. Winslow and Haywood (1931) have shown that changes in the viability of *E. coli* in salt mixtures can be accounted for by assigning to each cation a "specific potency." In reducing the respiration of *B. cereus,* calcium chloride is roughly three times as potent as sodium chloride, so that in terms of specific potency a solution containing xM NaCl + yM $CaCl_2$ is equivalent to a solution

containing $(x + 3y)M$ NaCl. Table 2 includes values for respiration in salt mixtures, calculated on this basis. They indicate approximately the degree to which respiration was inhibited in the various solutions. The rates of respiration in solutions with much calcium were usually lower than those indicated by calculation, perhaps because the specific potency assigned to calcium was low. (In this investigation the specific potency depended on concentration, being nearer to 10 than to 3 for calcium in dilute solutions): the calculation completely failed to indicate the changes in dilute solutions, and it appears that in them the specific potency concept was not applicable. It is clear, however, that the effects of concentrated solutions can be fairly satisfactorily accounted for in terms of the individual effects of the constituent salts. This emphasises the contention of Winslow and Haywood that the antagonisms between ions in their action on higher organisms do not exist when the same ions act on bacteria.

V. SUMMARY

From the results of manometric measurements of the rate of uptake of oxygen by cells of *Bacillus cereus* suspended in salt solutions, the following conclusions may be drawn.

1. The important factor in the determination of rate of respiration is the concentration of salt present.

2. All salts increase the rate of respiration when present in low concentrations, and decrease it in higher concentrations, unless special specific toxic properties of certain ions intervene.

3. This action of salts cannot be attributed to their ability to change the osmotic properties of the environment of the cells.

4. The increased respiration by unbuffered dilute saline suspensions is brought about by the cation of the salt: the salts of mono-, di- and tri-valent cations are equally effective at concentrations roughly in the ratio (1000:100:1).

5. With buffered suspensions of pH 6, this stimulating action of salts is increased slightly, probably because of the rather greater acidity of the buffered suspensions.

6. In unbuffered suspensions with high concentrations of salt, the rate of respiration is reduced according to the equation

$$\log r = P - Q.c.$$

7. Comparison of the inhibitory powers of different salts suggests that the inhibition of respiration is probably caused by the cations, and not by the anions.

8. There are no antagonistic effects between the ions $Na+$ and $K+$, and there is slight interaction between $Na+$ and $Ca++$, but only in dilute solutions.

9. With suspensions in phosphate buffer solution, initially of pH 6, the rates of respiration are lower in the presence of high concentrations of salts than those which would agree with the equation given in paragraph (6) above: this probably arises from an interaction between the salts and the buffer, which makes the suspensions considerably more acid than pH 6.

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NUTRIENT REQUIREMENTS OF BUTYRIC ACID—BUTYL ALCOHOL BACTERIA

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Preliminary investigations have shown that *Clostridium acetobutylicum* and related butyric-acid butyl-alcohol bacteria, fail to develop in a synthetic medium containing only ammonium sulphate, inorganic salts and glucose. These results suggest that perhaps the organisms require complex nitrogen compounds or some specific growth factor.

Wood, Tatum and Peterson (1937), Wood, Andersen and Werkman (1938) and Tatum, Wood and Peterson (1936) reported that an acidic ether-soluble factor extracted from Difco yeast extract and thiamin stimulate growth of propionic acid bacteria in an ammonium sulphate medium. Wood, Andersen and Werkman (1937a) (1937b) and Andersen, Wood and Werkman (1938) found that these stimulants were essential for growth of certain heterofermentative lactic acid bacteria in media containing hydrolyzed casein or purified amino acids. Andersen, Wood and Werkman (1937) described a homofermentative lactic-acid organism utilizing ammonium sulphate in the presence of lactoflavin, thiamin and the ether-soluble extract of yeast extract. The present investigation is a continuation of these studies using butyric-acid butyl-alcohol bacteria. Preliminary results (Brown, Wood and Werkman (1938a, 1938b) have shown, in media containing ammonium sulfate, glucose and Speakman's salt, that an ether extract of yeast extract is essential for growth of the butyl-alcohol bacteria. The yeast factor is not essential

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when the medium contains hydrolyzed casein. Recently McDaniel, Woolley and Peterson (1939) have accomplished considerable purification of the ether-soluble factor.

EXPERIMENTAL

Cultures

The organisms employed were five members of the genus, *Clostridium*, *C. acetobutylicum*, (I-2C), (12B), (1D), (B-1C), *C. felsineum* (5D), *C. beijerinckii* (4D), *C. butylicum* (53) and *C. pectinovorum* (10C). The cultures were purified by plating on peptone yeast-extract glucose agar under anaerobic conditions. Tubes of 5-per-cent corn mash were inoculated from well isolated colonies and shocked at 100°C. for 90 seconds.

Medium and analytical methods

Experiments were arranged to test the stimulating effect of various growth-promoting substances on the dissimilation of glucose in media containing hydrolyzed casein, amino acids and ammonium sulphate as sources of nitrogen. The compositions of the various test media are given in table 1. The constituents, unless otherwise given, were in the following concentrations: hydrolyzed casein 0.15 per cent prepared from Glaxo-casein A/E by sulfuric acid hydrolysis, ammonium sulphate 0.5 per cent, ether extract of Difco yeast extract 0.3 units per 10 ml. of medium (1 unit is the extract of 1 gram of yeast extract prepared as described by Wood, Tatum and Peterson, 1937), thiamin 0.1 gamma per 10 ml., riboflavin 0.01 mgm. per 10 ml., cystine 0.45 mgm. per 10 ml., tryptophane 1.6 mgm. per 10 ml. of medium. Speakman's salts: K_2HPO_4 0.025 per cent, KH_2PO_4 0.025 per cent, $MgSO_4 \cdot 7H_2O$ 0.01 per cent, NaCl 0.005 per cent, $FeSO_4 \cdot 7H_2O$ 0.005 per cent, $MnSO_4 \cdot 4H_2O$ 0.005 per cent. The percent given for each salt represents the final concentration in the medium. The mixture of amino acids[2] contained glycine, dl-alanine, dl-valine, dl-phenylalanine, l-tyrosine, l-proline, l-hydroxyproline,

[2] Amino acids purchased from the University of Illinois, Department of Chemistry and Eastman Kodak Co.

d-arginine, dl-lysine, l-histidine, l-cystine, dl-leucine, dl-isoleucine, threonine, dl-methionine, l-tryptophane, dl-serine and d-glutamic and l-aspartic acids. Each of these amino acids was used in final concentration of 0.00375 per cent on the basis of the naturally occurring isomer with the exception of cystine which was used in a concentration of 0.0015 per cent. Other accessory substances were used in the following concentrations: 30 gammas of nicotinic acid, 30 gammas of β-alanine, 90 gammas of uracil, 9 gammas of pimelic acid, and 3 gammas of pantothenic acid per 10 ml. of medium. The ether extract of hydrolyzed casein (prepared by extracting 1.9 grams of hydrolyzed casein and diluting to 100 ml. after removing the ether) was used in a concentration of 1 ml. per 10 ml. of medium. The residue from ashing a sample of ether extract of yeast extract was used in a concentration equivalent to 1 unit of ether extract per 10 ml. of medium. The filtrate from the acid hydrolysis of the ether extract of yeast extract was likewise used in a concentration equivalent to 1 unit of the original extract per 10 ml. of medium.

The various test media were prepared with distilled water, adjusted to pH 7.0, tubed in 10 ml. portions and autoclaved at 15 pounds pressure for 15 minutes. The inoculum used in each series of transfers was composed of 3 drops of a 48-hour culture of the organism in a broth composed of 0.5 per cent yeast extract, 0.1 per cent K_2HPO_4 and 1 per cent glucose. The cultures were *incubated for 3 days at 37°C. under vaseline seals. Growth was* measured in successive transfers by quantitative determination of glucose before and after fermentation, using a modification of the Munson and Walker method for reducing-sugar. In each series the successive transfers were made at intervals of 48 hours. Growth was considered positive when an appreciable amount of glucose was utilized in each of at least 5 successive transfers. Where quantitative analyses of the products of glucose dissimilation were made, 250 ml. fermentations in the test media were employed. For these, the inoculum consisted of 1 ml. from a third successive transfer of the organism in tubes of the test medium. Butyric and acetic acids were determined according to the method of Osburn, Wood and Werkman (1933), butyl and ethyl alcohols

by the method of Stahly, Osburn and Werkman (1934) and acetone by iodoform-titration as given by Wendell (1931).

Nutritional requirements in media containing hydrolyzed casein

The constituents essential in a basal medium of hydrolyzed casein were first determined. From a complex medium (no. 1, table 1) each constituent was singly eliminated and it was found that only Speakman's salt mixture was essential to growth (cf. no. 2, table 1). In fact, removal of all other constituents (ammonium sulfate, ether extract of yeast extract, thiamin and riboflavin) had no appreciable effect. *C. acetobutylicum* and *C. felsineum* grew in this medium of hydrolyzed casein, glucose and salt mixture. However, when potassium phosphate was eliminated from the medium (removed from Speakman's salt mixture) growth soon stopped in serial transfers owing probably to the necessity of phosphate in carbohydrate dissimilation. When phosphate was added and all other inorganic salts omitted, growth occurred. Apparently the hydrolyzed casein was not sufficiently free of salts to cause a deficiency other than in the case of phosphate which is used in relatively large quantities in cellular metabolism. When ether extract of yeast extract was added to the hydrolyzed casein-phosphate medium there was no increase in growth (nos. 5 and 6). The implication is that *C. acetobutylicum* will grow in a medium containing only amino acids, inorganic salts and glucose. Growth in media in which purified amino acids replaced hydrolyzed casein was then studied.

Nutritional requirements in a medium containing amino acids

A mixture of 19 amino acids containing those present in casein with the exception of hydroxyglutamic acid was found partially to replace hydrolyzed casein in a medium containing glucose and inorganic salts (nos 7 and 8).[3] Although less glucose was fermented than in the hydrolyzed casein medium, growth was maintained throughout 8 successive transfers. One or more factors may account for the reduced growth on the amino acid mixture. Hydroxyglutamic acid or some unknown amino acid

[3] Similar results were obtained with *C. felsineum*.

TABLE 1

Growth of Cl. acetobutylicum in test media

NUMBER	COMPOSITION OF MEDIUM*	GROWTH	INITIAL GLUCOSE	NUMBER OF TRANSFERS: 1	2	3	4	5	6	7
			mgm.	Mgm. glucose fermented per 10 ml. of medium						
1	H.cas.; NH_4; ET_2O ext.; salts; B_1; Tr.; Fl.	+	117.7	111.3	67.8	56.9	61.5	48.0	36.8	49.1
2	H.cas.; NH_4; ET_2O ext.; B_1; Tr.; Fl.	−	115.2	86.4	0.0					
3	H.cas.; salts	+	134.4	134.4	102.4	118.8	57.6	80.6	62.6	53.2
4	H.cas.; salts without PO_4	−	106.8	94.0	57.0	26.8	0.0			
5	H.cas.; PO_4	+	106.8	100.4	73.0	106.8	65.2	89.0	76.2	56.4
6	H.cas.; ET_2O ext.; PO_4	+	100.4	99.8	52.4	50.6	49.2	51.8	57.6	53.2
7	Amino ac.; salts	?	146.5	103.6	41.6	130.5	135.0	43.5	8.9	0.0
8	Amino ac.; NH_4; salts	+	133.1	45.4	21.1	37.1	37.1	30.7	24.3	35.8
9	Amino ac.; ET_2O ext.; salts	+	144.0	119.1	128.0	126.8	128.7	130.0	124.8	131.2
10	Amino ac.; NH_4; salts; B_1; Fl.; Access. sub.	+	118.4	30.8	19.2	25.6	17.3	24.4	22.4	21.8
11	Amino ac.; salts; B_1; H.cas. ext.	+	112.6	52.2	21.8	21.8	23.0	23.7	20.5	23.0
12	Cystine; NH_4; salts	−	134.4	22.4	9.6	0.0				
13	Asparagin; NH_4; salts	−	136.3	33.9	11.5	0.0				
14	NH_4; ET_2O ext.; salts; B_1; Tr.	+	115.2	67.2	66.0	67.2	78.1	57.6	55.1	44.2
15	NH_4; salts; B_1; Tr.	−	120.4	88.4	12.4	5.2	0.0			
16	NH_4; ET_2O ext.; salts	+	145.9	126.1	132.9	128.7	129.3	125.6	130.6	129.3
			113.2	78.0	86.4	57.0	58.8	69.2	106.8	68.4
17	NH_4; ET_2O ext.; salts without PO_4	−	115.2	108.8	102.4	0.0				
18	NH_4; ET_2O ext.; PO_4	−	113.8	16.6	11.4	0.0				
19	NH_4; salts; ash	−	160.6	5.1	7.4	0.0				
20	NH_4; salts; B_1; H.cas. ext.	−	139.5	26.9	24.3	0.0				
21	NH_4; salts; Access. sub.	−	133.7	37.7	13.4	0.0				
22	NH_4; salts; H.ET_2O ext.	+	102.4	83.2	82.0	84.5	85.8	80.7	83.2	87.7

H.cas. = hydrolyzed casein. NH_4 = $(NH_4)_2SO_4$. Salts = Speakman's salts. ET_2O ext. = ether extract of yeast extract. B_1 = thiamin. Fl. = riboflavin. Tr. = tryptophane. PO_4 = KH_2PO_4 and K_2HPO_4. Amino ac. = 19 amino acids. Access. sub. = mixture of nicotinic acid, β-alanine, uracil, pimelic acid, pantothenic acid. H.cas. ext. = ether extract of hydrolyzed casein. Ash = ash of ether extract of yeast extract. H.ET_2O ext. = hydrolyzed ether extract of yeast extract.

* All media contained glucose.

contained in casein may be beneficial, the amino acids in the mixture may not be present in optimal concentration or the hydrolyzed casein may contain accessory growth factors or inorganic salts not supplied by the Speakman mixture. Results in medium no. 9 show clearly that ether extract of yeast extract has a marked stimulating effect in the amino acid medium. However, when available accessory factors thiamin, riboflavin, nicotinic acid, β-alanine, uracil, pimelic acid and pantothenic acid were tested for stimulation, they had no influence comparable to that of ether extract (compare 9 and 10). The superior growth in hydrolyzed casein medium as compared to the amino acid medium suggested that the hydrolyzed casein contained some of the ether-soluble factor. Therefore an acidified sample of hydrolyzed casein was extracted and the extract tested. The results with medium 11 show the extract to be ineffective and indicate that hydrolyzed casein does not contain the ether-soluble growth stimulant or that the factor is in a combination which is ether-insoluble. The known influence of asparagin (Tatum, Peterson and Fred, 1935) and Brown, Stahly and Werkman, 1937) on growth of the butyl alcohol bacteria and of cystine for other bacteria, made it advisable to determine whether these substances can replace the amino acid mixture (12 and 13). Both were ineffective.

Nutritional requirements with ammonium sulfate as a nitrogen source

It was established that in a medium containing ammonium sulfate, ether extract of yeast extract, Speakman's salts, thiamin, tryptophane and glucose, growth was vigorous (no. 14). When the ether extract of yeast extract was omitted there was no growth in the fourth transfer (no. 15). The omission of thiamin and tryptophane caused no decrease in growth (no. 16). Medium 16 consisting of ammonium sulfate, Speakman's salts, ether extract of yeast extract and glucose, is free of amino acids, yet supports vigorous growth of the butyl alcohol bacteria. Additional results with a number of other cultures are shown in table 2 in which growth in the ammonium sulfate medium is compared to that obtained in yeast extract medium. The growth was as

TABLE 2

Comparative fermentation of glucose in $(NH_4)_2SO_4$ and yeast extract media

CULTURE NUMBER	MEDIUM†	NUMBER OF TRANSFERS									
		1	2	3	4	5	6	7	8	9	10
		Mgm. initial glucose per 10 ml.									
	$(NH_4)_2SO_4$	113.2	113.2	84.4	84.4	84.4	84.4	84.4	112.0	112.0	112.0
	Yeast extract	149.8	149.8	102.4	102.4	102.4	102.4	102.4	102.4	102.4	102.4
		Mgm. glucose fermented per 10 ml.									
4D	$(NH_4)_2SO_4$	17.2	63.6	58.8	84.4*	78.0	49.2	49.2	75.0	68.6	62.8
4D	Yeast extract	89.0	85.2	35.2	102.4	83.2	57.6	65.4	35.2	35.2	35.2
53	$(NH_4)_2SO_4$	110.0	113.2*	84.4*	84.4*	84.4*	84.4*	84.4*	112.0*	112.0*	112.0*
53	Yeast extract	146.6	146.6	102.4*	89.6	102.4*	102.4*	102.4*	102.4*	38.4	59.0
10C	$(NH_4)_2SO_4$	20.0	78.0	47.4	40.4	47.4	39.6	34.6	51.2	49.4	51.2
10C	Yeast extract	75.0	74.4	6.0	38.4	57.6	52.6	38.4	38.4	102.4*	59.0
1D	$(NH_4)_2SO_4$	67.2	113.2*	84.4*	84.4*	84.4*	84.4*	84.4*	112.0*	112.0*	112.0*
1D	Yeast extract	137.0	149.8*	67.2	99.2	89.6	102.4*	102.4*	102.4*	102.4*	16.8
12B	$(NH_4)_2SO_4$	113.2*	100.4	84.4*	84.4*	84.4*	84.4*	84.4*	112.0*	112.0*	112.0*
12B	Yeast extract	149.8*	149.8*	73.6	102.4*	102.4*	102.4*	102.4*	102.4*	102.4*	102.4*
5D‡	$(NH_4)_2SO_4$	129.9	131.2	126.7	134.4	133.1	130.6	129.9	129.3	133.1	132.9
5D§	Yeast extract	121.6	121.6	122.3	121.6	121.6	121.6	120.4	122.9	121.6	118.4

* Glucose completely fermented.

† $(NH_4)_2SO_4$ medium: Ether extract, Speakman's salts, ammonium sulphate, glucose, pH 7.0. Yeast extract medium: Difco yeast extract, glucose, dipotassium phosphate, pH 7.1.

‡ Mgm. initial glucose = 145.9 mgm. per 10 cc.

§ Mgm. initial glucose = 128.0 per 10 cc.

vigorous with ammonium sulfate as with yeast extract. It is significant that medium 16 was the most satisfactory of all the media tested. Results with medium no. 17 show that phosphates are essential for growth and no. 18 that in addition other inorganic salts of the Speakman mixture are required. In order to establish definitely that the ether extract contains an essential organic factor it was ashed and the resulting product was added to medium 19 in place of the complete extract. The results show the factor is organic, i.e., not replaceable by the ash. There was

TABLE 3

Products of the fermentation of glucose by butyric-acid butyl-alcohol bacteria

MEDIUM	CULTURE NUMBER	GLUCOSE FERMENTED	GLUCOSE FERMENTED PER LITER	PRODUCTS PER 100 mM OF GLUCOSE FERMENTED				
				Butyl alcohol	Acetone	Ethyl alcohol	Butyric acid	Acetic acid
		per cent	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Hydrolyzed casein*	12B	100	111.0	53.1	11.7	11.7	18.4	35.7
	B-1C	91	101.0	58.1	29.7	10.8	14.8	31.7
	5D	55	63.3	17.6	18.3	28.7	36.3	39.3
$(NH_4)_2SO_4$*	1-2C	94	59.0	14.7	17.9	17.3	35.1	44.6
	12B	99	62.0	4.2	15.8	19.2	56.0	31.2

* Hydrolyzed casein medium contained hydrolyzed casein 0.18 gram, $(NH_4)_2SO_4$ 0.6 gram, ether extract of 1.5 grams of yeast extract, thiamin 2.0 gammas, tryptophane 10 mgm., K_2HPO_4 0.1 gram per 100 cc. of medium.

$(NH_4)_2SO_4$ medium contained $(NH_4)_2SO_4$ 0.5 gram, ether extract of 3 grams of yeast extract per 100 cc. of medium plus Speakman's salts.

no growth in medium 20 in which the ether extract of yeast extract was replaced by ether extract from hydrolyzed casein. Likewise nicotinic acid, β-alanine, uracil, pimelic acid, and pantothenic acid were unable to replace the ether extract (no. 21).

If hydrolyzed casein contains a factor other than amino acids it must be stable to acid hydrolysis. To determine the stability of the factor in ether extract of yeast extract a sample was autoclaved at 20 pounds pressure for 3 hours in N sulfuric acid. The solution was neutralized with sodium hydroxide and substituted for the ether extract. The activity of the factor was not altered by the acid treatment (medium 22). If the ether-soluble factor

or some combination of the factor is present in hydrolyzed casein, it should be stable to the acid hydrolysis.

Data from the analyses of fermentations in which hydrolyzed casein or ammonium sulfate were used as nitrogen sources are shown in table 3. The normal products of dissimilation were formed from glucose; however, the yield of butyl alcohol was low in ammonium sulfate medium.

SUMMARY AND CONCLUSIONS

An investigation of the growth factor requirements of butyric-acid butyl-alcohol bacteria showed:

1. An ether-soluble extract of "Difco" yeast extract and of Speakman's salts are essential for growth in media containing ammonium sulfate as a source of nitrogen. Neither the Speakman's salts minus the phosphates nor the phosphates without the other inorganic salts are sufficient for growth in successive transfers. The ether-soluble yeast factor is not altered by prolonged heating in N sulphuric acid.

2. A number of substances known to be growth factors for organisms did not replace the extract of yeast extract for growth of butyric-acid butyl-alcohol anaerobes. These substances include thiamin, riboflavin, tryptophane, cystine, asparagin, nicotinic, pimelic and pantothenic acids, β-alanine and uracil. These substances do not stimulate utilization of glucose when added to media containing an amino acid mixture.

3. In the presence of purified vitamin-free hydrolyzed casein as a source of nitrogen and glucose as a source of carbon, phosphates alone were essential for growth. Other inorganic salts were undoubtedly included in the hydrolyzed casein. An ether-soluble growth stimulant could not be obtained or demonstrated in the hydrolyzed casein. The growth-promoting property of hydrolyzed casein appears to be a function of the amino acids. This is substantiated by the fact that a mixture of 19 purified amino acids naturally present in casein are sufficient for growth when supplemented with ammonium sulphate and Speakman's salts. In this case growth of the butyric-acid butyl-alcohol bacteria has been obtained in a medium of known composition.

4. Glucose is dissimilated with the formation of normal products in media containing ammonium sulphate or casein hydrolysate as source of nitrogen when the other essential nutrients are present.

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CYTOLOGY AND METHODS OF REPRODUCTION OF TWO COCCI, AND THE POSSIBLE RELATION OF THESE ORGANISMS TO A SPORE-FORMING ROD

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During an investigation into the life-cycle of a spore-forming *Bacillus* (whose cytology has been described elsewhere (Allen, Appleby and Wolf, 1939)) this organism was grown in aerated broth cultures. A jar containing about 800 ml. of inoculated broth was fitted with a tight rubber bung coated with pitch, and connected both with a water pump and with a flask of 1 per cent $HgCl_2$ through which air was admitted. Control flasks of uninoculated broth inserted between the culture jars and $HgCl_2$ flasks acted as indicators of the sterility of the incoming air. A piece of glass tubing passing through the bung of the culture jar and connected with the pump provided the air outlet, and was fitted with a tight plug of cotton wool. The essential part of the apparatus was immersed in a thermostat at the optimum temperature of the inoculated spore-former. After about four weeks' incubation in this manner cocci appeared in the cultures, sufficiently numerous to isolate by plating dilutions of the cultures. These cocci were of two types—yellow pigmented sarcinae, and non-pigmented micrococci. These organisms showed in living preparations variations in morphology more complex than are usually attributed to cocci, which were not revealed by examination of fixed and gram-stained films. It became evident by vital staining methods that these cocci possessed internal structures, and were capable of methods of reproduction other than transverse fission. A careful investigation of these appearances throughout the life of a culture was therefore undertaken for the purpose of determining the nature of the cytological changes

related to growth, in what morphological forms these organisms were capable of existence, and whether any relation to the original spore-former was evident under ordinary cultural conditions.

METHODS OF PURIFICATION AND EXAMINATION

Before examination, both these cocci were subcultured many times at short intervals, and proved to be very stable. Strains from different aerated cultures of the spore-forming rod were purified by six platings, followed by single cell isolations by Gardner's method on a very thin slab of clear nutrient agar. Twelve-hour broth cultures were used as the inoculum for nutrient agar slope cultures for microscopic examination. For each series of examinations two sets of slopes were similarly inoculated, one set being twelve hours older than the other, so that by examination of slopes of both sets during twelve hours each day the whole of the period of development of the organism was covered.

Microscopic examinations were made constantly during several weeks by a modification of Nakanishi's vital staining method (Stoughton, 1929). Of a variety of different basic stains used, one—neutral red chloride—gave the clearest results. Preparations were examined immediately, and also after the organisms had been in contact with the stain for a few hours.

THE SARCINA

Characters of the sarcina

The *Sarcina* grew well aerobically on nutrient agar and in nutrient broth at 30°C., producing a yellow pigment. In gram-stained preparations, a 48-hour culture had the appearance of a typical gram+ *Sarcina,* but there was considerable variation in the size of the cells, the majority being rather large.

In nutrient gelatin stab cultures there was filiform growth followed by slow liquefaction after 3 weeks at 20°C., at first saccate but becoming stratiform.

No fermentation occurred in any carbohydrate broths until after a minimum period of 11 days' incubation at 30°C., after which acid without gas was formed from glucose, maltose and

sucrose. After 4 to 5 weeks' incubation, acid was formed in addition in xylose, arabinose, raffinose, dextrin, inulin and starch broths. (It may be a fact of significance that these particular carbohydrate reactions then corresponded with those of the original spore-forming rod after a few days' incubation.)

The thermal death point of the organism, determined by the percentage survival method of Esty and Williams (1924) with 4-day cultures, was 58.8°C. for 15 minutes.

Morphology and cytology of the sarcina

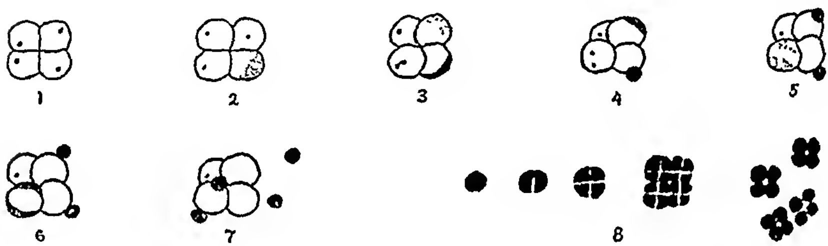
An 8 to 16 hour growth of the organism consisted of large dense clumps, roughly cubical in shape, composed each of probably several hundred individuals. The cells were flattened, as if tightly pressed together. These clumps were presumably due to the fact that at this stage of growth division was far more rapid than the separation of individuals, but at 16 hours and onwards the groups gradually became smaller. The cells finally became separated out into regular cubical groups of four and eight, with some pairs and single individuals.

The large clumps of cocci in young preparations stained very deeply, but as they separated out into small groups the cytoplasm stained much less readily and intracellular granules became apparent. The majority of cells contained one round densely staining granule, generally situated near the cell membrane, and less often centrally. It appeared to divide by elongation and constriction in the middle, presenting temporarily a dumb-bell shaped form, and resulting in two round granules in one cell. This was probably an immediate preliminary to cell division. Granules apparently similar to these in form and behaviour—whose division is related to cell division—have been described by many other workers in species of rods and cocci. They were found in cells of *Phytomonas malvacearum* by Stoughton (1929) in two aerobic spore-formers by Badian (1933), and Dobell (1911) demonstrated similar granules in representatives of many different types of bacteria. These workers concluded that such granules probably represented nuclei.

Though the most common method of reproduction of this

coccus was by transverse fission, budding was also very evident by vital staining methods. The formation of a bud appeared to be preceded by the appearance of many small granules situated in one half of the cell, while the cytoplasm of the other half remained clear. These granules collected near the cell membrane forming a crescent-shaped structure which stained very deeply. This finally bulged outwards and formed a round densely stainable body attached to the larger and feebly stained parent cell. Such appearances as are represented in figures 1–7 were a conspicuous feature in preparations throughout the whole period of growth of the culture.

Less frequently, one cell gave rise to two buds at the same time. It appeared that this occurred when bud formation took place



FIGS. 1–8. DIAGRAMMATIC REPRESENTATION OF THE FORMATION AND MULTIPLICATION OF BUDS OF THE SARCINA

immediately after a division of the nucleus-like body and transverse fission (normally following this division) failed.

The buds after separation multiplied, so far as could be determined, solely by transverse fission in three planes, giving rise to very regular cubical groups of minature sarcinae which stained very deeply (fig. 8). There was thus to be seen in the culture a sharp distinction into two types of individuals—the large feebly stained cells with a nucleus-like structure, and densely stained miniature counterparts. The latter, however gradually increased in size and became similar to the parent, thus completing a growth cycle. The only indication of these events in gram-stained preparations was the variation in size of the individuals.

After 4 days' incubation, a large number of cells contained one fairly large spherical acid-fast granule, and similar granules were

found free. These did not undergo any evident change after liberation during several weeks. Though spore-like in appearance they were not heat resistant.

Minute exogenous gonidia, projecting from the membrane on slender stalks, were very numerous, particularly in cultures about 24 hours old. At this age on one cell might be seen several of these gonidia. In older cultures the number decreased, and they were not found at all after 4 days. These gonidia were probably the origin of many small granules found free which stained very deeply, and showed active Brownian movement. They may have accounted for the appearance in these cultures of minute slender rods, which were sometimes seen in large numbers. These rods were particularly evident after 12–36 hours' incubation when they were found in groups lying around the groups of cocci. In older cultures they increased in length to about 6μ, but were always very slender and generally granular. Though fairly abundant in young cultures these rods apprently multiplied much less readily than the cocci, and formed after a few days only a very small proportion of the total number of organisms. They could be found in small numbers during 18 weeks' incubation.

The significance of these small rods in the life-cycle of the cocci is not understood. In their appearance and behaviour they bear a striking resemblance to the small rod variant produced by the parent spore-forming rod which has been previously described (Appleby, 1939a), and which was shown to be capable of reproducing the parent type.

In cultures about 8 weeks old, forms resembling gonidangia appeared. A round structure with a diameter of about 5 to 6μ was apparently formed by fusion of a small number of adjacent cells, their contiguous membranes disappearing. The thick membrane of this spherical body stained very deeply. The cytoplasm was at first homogeneous and faintly staining, and surrounded by a narrow clear unstained layer. Gradually there appeared in the cytoplasm a number of small spherical granules, uniform in size, with an intense affinity for stain. The structure of these gonidangium-like forms is shown diagrammatically in

figures 9 to 12. From their large number, and very clear and uniform appearance, these structures were not either artefacts or decaying forms, but presumably a type of reproductive body homologous with the asci of yeasts, and resembling the gonidangia described by other workers as occurring in bacterial cultures.

THE MICROCOCCUS

Characters of the micrococcus

This organism was a gram+ species growing well aerobically on ordinary media at 30°C. and producing no pigment.

Growth in nutrient gelatin stab cultures was filiform, followed by slight saccate liquefaction after 3 to 4 weeks at 20°C. Acid without gas was produced from glucose, sucrose, lactose, maltose and arabinose after 2 days at 30°C., while galactose, raffinose, starch, dextrin, inulin, xylose and mannitol were fermented much

FIGS. 9–12. STAGES IN THE FORMATION OF A GONIDANGIUM-LIKE STRUCTURE

more slowly, acid only being apparent after 1 to 3½ weeks' incubation. In litmus milk, slight acid only was produced. Indol and acetyl-methyl-carbinol tests were negative during 3 weeks at 30°C. Nitrates were reduced, and the methyl red test was positive after 4 days at 30°C.

The thermal death point of 4 day cultures by the percentage survival method was 59.2°C for 15 minutes.

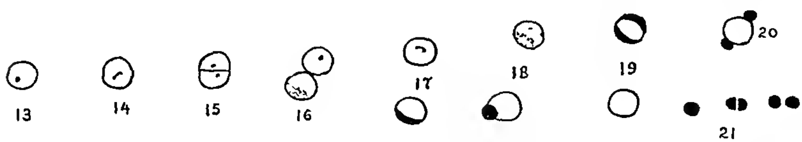
Morphology and cytology of the micrococcus

After a few hours' incubation, cultures of this organism consisted of short thick rods, staining feebly. During the period of active division these rods were reduced to spherical cells, and remained as such throughout the life of the culture. This tendency to exist at an early growth stage as a rod was most evident in freshly isolated strains of this organism, and diminished after repeated subculture.

In vitally stained preparations it was evident that there were two distinct methods of multiplication comparable with those of the *Sarcina*. These were apparent, though varying in frequency, throughout the whole of the period of growth. These were:

1. transverse fission, producing apparently identical individuals;

2. budding, in a manner exactly similar to that of the *Sarcina*, resulting in the formation of very small micrococci, easily dis-



FIGS. 13–21. FORMATION AND MULTIPLICATION OF BUDS OF THE MICROCOCCUS

FIG. 22

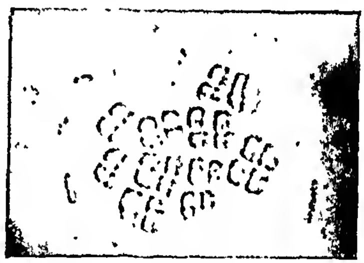


FIG. 23

FIG. 22. PHOTOMICROGRAPH OF SARCINAE SHOWING YOUNG, DEEPLY STAINED BUDS

FIG. 23. PHOTOMICROGRAPH OF A PREPARATION FROM A 12 HOUR CULTURE SHOWING SMALL RODS

tinguishable at first from the parent by their small size and greater staining capacity. One cell frequently produced two buds at the same time, diametrically opposed. The process of bud formation is represented diagrammatically in figures 13 to 21.

Internal nuclear structures were most clearly seen (as was also the mechanism of bud formation) on blood serum slopes where the organism grew very abundantly and the cells were particularly large. They contained each one granule, most often round, but sometimes in the form of a short bent rod, either uniform in width or thickened at the ends. In newly divided cells, before the

adjacent portions had become rounded off, the granules (one in each cell) were small and round. These nucleus-like structures were most evident in young cultures at a period of active multiplication.

Small rods were produced by this micrococcus, similar to those of the sarcina. They were much less numerous however, and though present in small numbers in cultures of all ages were absent from many microscopic preparations.

In cultures about 3 months old, when the medium was very dry and growth scanty, gonidangia were formed. These were round, thick-walled cells, about 3 to 5μ in diameter. When fully formed, they contained each a small number of small spherical granules.

THE POSSIBLE RELATION OF THESE COCCI TO A SPORE-FORMING ROD

The two cocci described were obtained from old aerated broth cultures of a spore-forming *Bacillus*. Though all due precautions were taken to prevent contamination during aeration it is not possible to state with certainty that these cocci were in reality products of growth of the spore-former. There is, however, some evidence to suggest that this may be so. As it has been shown by other workers (notably Löhnis and Smith (1916, 1923), Mellon (1919), Cunningham (1930) and Cunningham and Jenkins (1927), and Mellon, Richardson and Fisher (1932)) that some rod species may under certain conditions produce stable cultivable cocci, and as the species under investigation is capable of a pleomorphism greater than is commonly recognised, the facts which indicate a relationship in this case may deserve consideration.

As has been reported in a previous paper (Appleby, 1939b) stable cultures of the micrococcus have been obtained several times from filtrates of the bacillus, using the serial transfer method of cultivation, and four times from filtrates without serial transfer. These were mainly from non-aerated cultures.

On examination of recently isolated strains of both cocci from aerated cultures there were a few instances of apparent reversion—the cocci presumably giving rise to the original spore-

forming rod. In one slope culture of the sarcina, purified by repeated plating and single-celling, there appeared suddenly after $8\frac{1}{2}$ weeks' incubation spore-forming rods recognisable as those of the species originally used. When first apparent, these rods were distributed throughout the whole culture, on all parts of the slope, and therefore it appears unlikely that they were external contaminants. If they had represented a contaminant of the inoculum they would presumably have made their appearance much earlier.

In one similarly purified slope culture of the micrococcus there appeared a growth of the bacillus after $4\frac{1}{2}$ weeks' incubation. The wide distribution of the rods at their first appearance suggested an origin within the culture.

One purified culture of the micrococcus grown in a flask of about 300 ml. of broth produced after $4\frac{1}{2}$ weeks a large number of slender rods, similar to the rods found in small numbers in cultures of both cocci, and to the small rod variant of the bacillus investigated (Appleby, 1939a). These were followed by growth of the spore-forming rod. It may be therefore that the small rods produced by both cocci can function as an intermediate stage in the reversion of the cocci to the spore-former. In no cultures of these cocci was any foreign type of rod ever found, other than the spore-former originally used for this work, and a form similar to its small rod variant.

The majority of the coccus cultures examined—on a variety of media, in slope and shake cultures—remained purely as cocci, showing no morphological changes other than those associated with regeneration of cocci or their related small rods. The failure to obtain reversion in these cultures may have been due to the fact that nearly all examinations were made with strains previously subcultured many times on the same medium at short intervals—a procedure which might make for stabilisation of a variant type. No cocci when freshly isolated were examined in old cultures.

Subsequent attempts to reproduce these cocci from the bacillus under different environmental conditions have failed. It may be that after a few years' artificial cultivation on one particular

laboratory medium the life-cycle of an organism becomes modified, tending to a greater monomorphism than may be displayed by that organism in its natural habitat.

SUMMARY

Two species of cocci—a *Sarcina* and a *Micrococcus*—are described which (a) possess a nucleus-like granule, dividing before cell division; (b) are capable of multiplication not only by fission but by budding, and at certain growth periods by formation of endogenous (and in the case of the *Sarcina* also exogenous) gonidia. The formation and development of the buds constitutes a cyclical series of morphological changes; (c) are capable of producing very small slender rods.

There is some scant evidence to suggest that these cocci may be produced by, and capable of reversion to, a spore-forming rod.

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SOME VARIATIONS IN MORPHOLOGY OF A SPORE-FORMING BACILLUS

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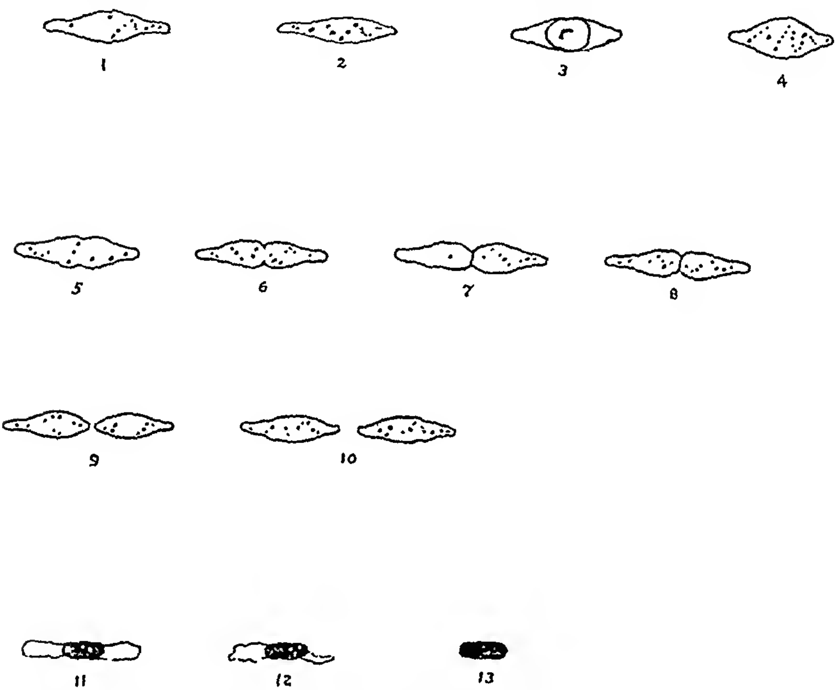
There is abundant evidence in bacteriological literature to show that a species may exhibit temporary changes in morphology in response to environmental conditions. Thus, with spore-forming species, sporulation may be inhibited on certain media and at high temperatures; cultivation under conditions slightly injurious to the normal type of cell may result in unusual morphological appearances; and peculiar forms in old cultures originally described as aberrant involution forms have frequently been shown to be capable of active reproduction, their unusual appearance being attributed to the changed conditions of the old medium. Growth and multiplication therefore are not restricted to the morphological form typical of the species.

An interesting change in morphology is shown by a strain of a spore-forming rod when grown at low temperatures, and this is accompanied by a complete change in the naked eye appearance of the growth on a solid medium. This organism appears in gram-stained preparations to be a typical member of the *Bacillus* genus, and the morphology at its optimum temperature of 30°C. has been described in a previous paper (Allen, Appleby and Wolf, 1939). Surface plate colonies on nutrient agar at 30°C. are large, greyish, flat, rough, papillated, with a spreading margin, and easily emulsifiable.

Nutrient agar plates inoculated with the organism were incubated 14 hours and then left at room temperature, in the dark, for 2½ weeks. The colonies were then well grown, but quite unrecognisable as belonging to the species inoculated. They had a slight yellow pigment, and surface colonies were raised, moist,

and waxy in appearance, of a slightly sticky consistency. The surface was smooth, sometimes radially striated, and the margin either entire or lobed.

Colonies were examined by vital staining using Nakanishi's method as modified by Stoughton (1929), and neutral red chloride. Only an occasional parallel-sided rod of the usual appearance was seen and no spores were formed. The majority of organisms were



FIGS. 1–13. MORPHOLOGY OF AN AEROBIC SPORE-FORMER GROWN AT ROOM TEMPERATURE

1 to 4, types of spindle-shaped bells; 5 to 10, method of fission of spindle-shaped bells; 11 to 13, form suggestive of zygospore formation.

large spindle-shaped cells, about 4 to 8 times the size of an ordinary rod of this species. These types are shown diagrammatically in figures 1 to 10. They multiplied by a process intermediate between fission and constriction, resulting in two equal parts, which by elongation at one pole became spindle-shaped again. They contained stainable granules of varying sizes which were usually arranged in the form of a loose spiral stretching right

across the cell, similar to the nuclear arrangements described by Dobell (1908) in cells of *Bacillus spirogyra*, *Bacillus flexilis* and *Bacillus butschlii.*

Some spindle-shaped cells, after about six weeks at room temperature, produced one large round intracellular body which stained feebly, often containing a nucleus-like structure, and was surrounded by a more deeply stainable membrane (fig. 3). As these structures were also found free they were presumably reproductive bodies. Though they had the size and outline of large spores they did not entirely lose their staining capacity.

There were also found in these colonies long thick threads, some showing rudimentary branches, and forms distinctly suggestive of conjugation. These were represented by a densely staining short rod at the junction between two apparently empty membranes, which shrivelled up and left the rod-like form free (figs. 10–13).

On subculture of these atypical colonies to a nutrient agar slope and incubation at 30°C. a change, culturally and morphologically, to the recognised species type occurred. After 12 hours' incubation there was fairly good growth resembling in appearance the yellowish, waxy, non-spreading atypical colony growth. But microscopic examination revealed a large number of ordinary parallel-sided rods, developing from the spindle shapes by elongation and segmentation. After 24 hours the growth was indistinguishable, both macroscopically and microscopically, from the normal growth.

One of these colonies of spindle-shaped organisms was rejuvenated by ten subcultures on nutrient agar, incubating at 30°C. It was then plated, incubated a short period, and kept at room temperature as before. A similar procedure was carried out with a different strain of the same organism. Colonies of the latter after 3 weeks had not noticeably increased in size since removal from the incubator, and the constituent cells were ordinary parallel-sided rods. The former, however, developed a much larger amount of growth, and after 8 days many of the normal rods were assuming spindle shapes by enlargement and swelling in the middle. They developed exactly as those described above.

Different strains of this species therefore differ in the readiness with which they adapt themselves to low temperature growth, where active multiplication in the normal rod form apparently cannot take place, and once this atypical growth has occurred it may readily be repeated.

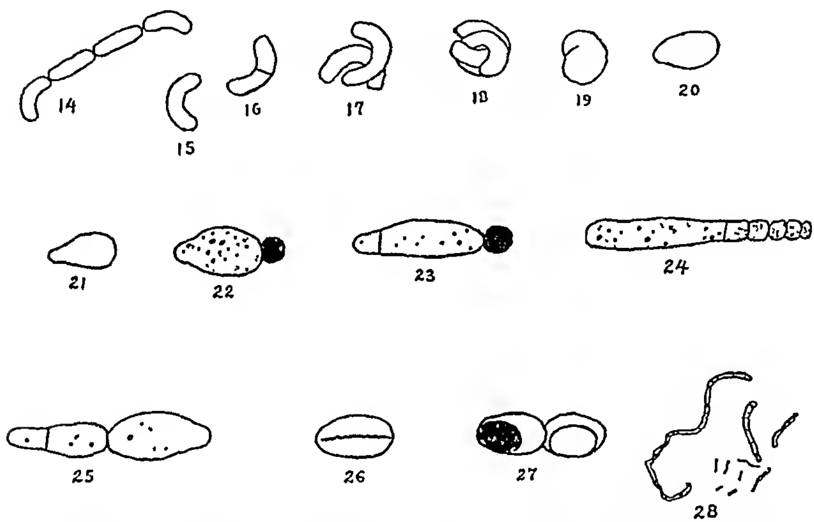
When this spore-forming rod is grown on inspissated blood serum slopes at 30°C. it may not be recognisable as a *Bacillus* without careful scrutiny. This is not due to the production on this medium of an entirely different type of cell (as occurs at low temperatures) but to the stimulation of certain of the peculiar forms found in old nutrient agar cultures, of the types previously designated involution forms, not generally recognised as a growth phase of a *Bacillus*.

The young growth on this medium presents the same features at 30°C. as a nutrient agar slope at this temperature, and after 2 days the culture consists largely of free spores. There then follows a period of active multiplication of some atypical forms found, in smaller numbers, in nutrient agar cultures, so that the free spores are far outnumbered by these growing types.

On examination of serum slopes by vital staining methods it was found that there were two morphological forms particularly in evidence in cultures more than two days old—structureless feebly staining curved rods, which appeared to be capable of conjugating in pairs and giving rise to bean-shaped structures (as represented in figs. 14–20); and threads and filaments of various dimensions, some very slender and twisted, and generally containing numerous granules. There were found also large irregular bulbous forms, multiplying by budding as well as fission into unequal sized portions (figs. 22–25). Some of these large forms contained a long central stainable structure, either composed of a string of small granules, or a uniform filament spirally twisted (fig. 26). Some of these cells were seen to be forming endospores in cultures 1 to 4 weeks old.

Very small slender rods were numerous on this medium throughout the whole of the period of growth. These were found in small numbers in nutrient agar cultures of this spore-former, and their isolation by filtration and growth in pure culture has been described in a previous paper (Appleby, 1939).

It is evident therefore that this spore-former is capable of such wide variation in morphology—under these two conditions of growth—as to be almost or completely unrecognisable as a species of the *Bacillus* genus, and growth and multiplication may be as abundant among these unusual cell forms as it is among the forms recognised for the genus. It would appear that the capacity of a species for growth in different morphological forms is a matter deserving consideration, since morphology is an important criterion in classification.



FIGS. 14–28. SOME OF THE MAIN MORPHOLOGICAL TYPES IN SERUM SLOPE CULTURES OF AN AEROBIC SPORE-FORMER

SUMMARY

A spore-forming rod, which in gram-stained preparations from nutrient agar at its optimum temperature of 30°C. exhibits the usual appearance of members of the *Bacillus* genus, has been found to exist in entirely different morphological forms under different growth conditions. At a low temperature, and on inspissated blood serum slopes, changes in morphology may occur very readily and be so complete as to render the organism temporarily unrecognisable. At room temperature there is simultaneously a change in the appearance of the growth.

The unusual cell forms described (including types reported by early bacteriologists to be involution forms) are capable of active multiplication, and show, in vitally stained preparations, reproductive processes other than binary fission. That they are the result of environmental conditions is shown by the fact that these forms readily return to the normal parallel-sided spore-forming rod type when cultivated on nutrient agar at 30°C.

The author wishes to express her thanks to Dr. L. A. Allen for his advice during the conduct of this work.

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THE NATURE OF THE CATALASE REACTION IN THE RESIDUE OF STAPHYLOCOCCUS AUREUS LYSED BY BACTERIOPHAGE[1]

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There is considerable interest in the problem of the possible respiration of active bacteriophage particles because of the bearing that its solution would have in establishing the living or non-living nature of this virus-like agent. Bronfenbrenner (1924, 1926 a and b), employing an indicator method sensitive to 0.02 milligram of carbon dioxide, was unable to detect carbon dioxide production by phage particles in experiments extending over several hours. Wohfeil (1928) also was unable to detect carbon dioxide production by the phage particles and Bachmann and Wohlfeil (1927) could detect neither carbon dioxide elimination nor oxygen use by phage particles in the presence of dead cells of sensitive strains.

McKinley and Coulter (1927) detected a slight increase in the amount of carbon dioxide produced by a strain of *Escherichia coli* grown in the presence of bacteriophage, but these authors considered this increase to be due to an increased growth rate of the bacteria rather than to any metabolic activity of the phage particles themselves.

Parker and Smythe (1937) could detect neither carbon dioxide production nor oxygen use by concentrated preparations of vaccine virus. This result was not due to the lack of a suitable

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oxidizable substrate, since negative results were also obtained in the presence of glucose and glucose monophosphate.

On the other hand, Eaton (1931) carried out an extensive study on lysed *Staphylococcus* cultures by manometric methods and reported an observable uptake of oxygen and production of carbon dioxide. He pointed out that the oxygen consumption possibly might be due to a purely chemical autooxidation of some component present in the medium but that the carbon dioxide production indicated metabolic activity. Following the work of Eaton, we have again carried out a great many experiments to detect the oxygen consumption of lysed cultures of *Staphylococcus* by a manometric method sensitive to 0.0002 of a milligram of oxygen. Even solutions containing 10^{10} particles of phage per milliliter uniformly gave negative results.

A superficially analogous result is observed in the metabolism of red blood cells, lysed by distilled water. Although the intact cells have a readily observable oxygen consumption, this metabolic activity is lost in lysis. In this instance it is known that lysis inhibits the mechanism of sugar phosphorylation, and that this power of oxidation by molecular oxygen is regained if the proper glucose-phosphate ester is added.

It is evident that biological oxidation mechanisms do not depend intrinsically on the presence of structurally intact cells. The analogy with red blood cells suggests that the loss of the power to use molecular oxygen by bacterial cells lysed by bacteriophage is due to the disruption of some necessary enzymatic complex. This is all the more probable in view of the work of Hetler and Bronfenbrenner (1928) who showed that there is an hydrolysis of bacterial protein during lysis as shown by the increase of free amino nitrogen. Bronfenbrenner and Muckenfuss (1927) have shown that even dead bacterial protein may undergo hydrolysis by enzymes released during the lysis of the living cells by the bacteriophage. The cessation of oxygen consumption could be explained if the proteinaceous carriers of the prosthetic groups of enzymes are destroyed, or modified, or separated from their respective active groups during the process of lysis.

The present study was undertaken to determine the effect of

the lysis of bacterial cells by bacteriophage on the activity of a specific enzymatic complex. Catalase was the enzyme selected for study because of the great accuracy with which the evolution of oxygen from hydrogen peroxide can be observed by the use of the Barcroft differential manometer in a constant-temperature water bath. The relation of catalase to the respiratory process is still a matter of conjecture. Nevertheless, a study of the effect of bacteriophage on this enzyme should furnish interesting data on the ability of bacteriophage to disrupt a specific enzymatic complex.

Schuller (1935) tested for the activity of a number of enzymes in the centrifuged lysed culture of *E. coli.* Negative reactions were obtained for trypsin, papain, lipase, amylase, maltase, nucleosidase, urease, arginase and catalase. Only phosphatase gave a positive reaction. His paper contains, unfortunately, only scanty details of his experimental procedure. On the other hand Lominski (1934) reported that lysed cultures exhibited catalytic activities proportional to the number of cells that had undergone lysis.

Fejgen, Bronislawa and Supniewski (1923) were not able to detect catalase nor reductase in lysed cultures, and on that account they concluded that the lytic agent was non-living.

EXPERIMENTAL PROCEDURE

The 18-hour growth of *Staphylococcus aureus* on agar was suspended in 10 ml. of broth. This suspension was filtered through sterile cotton and 0.1 ml. of the filtrate was added to 10 ml. of broth. Then 1 ml. of a bacteriophage solution, containing 10 active particles per ml. was added. The final dilution, therefore, contained about one particle of phage per milliliter. After a few hours of incubation at 37°C., the lysis was complete and the suspension became crystal clear. This lysed culture was then filtered through a Berkefeld filter and tested for active catalase by observing the evolution of oxygen from hydrogen peroxide in Barcroft differential manometers. In all instances, the hydrogen peroxide was tipped into the solution of lysed cells from a side arm, after temperature equilibrium had been reached.

RESULTS

Experiment 1

The arrangement and the results of experiment 1 are indicated in table 1 and figure 1. Since control broth caused no detectable evolution of oxygen from hydrogen peroxide and lysed cells

TABLE 1

The rate of liberation of O_2 from H_2O_2 by the filtered lysate in cubic millimeters at the time indicated

	MANOMETER 1 (CONTROL BROTH)	MANOMETER 2 (FILTRATE + KCN)	MANOMETER 3 (FILTRATE)
Buffer (pH = 8.14)..........		2.0 ml.	2.0 ml.
Filtrate of lysed culture		0.2 ml.	0.2 ml.
2 per cent KCN at pH = 8.14		1.0 ml.	
Broth.....................	10 cc.		
3 per cent N_2O_2 at pH = 8.14.	2 cc.	2.0 ml.	2.0 ml.
Time	O_2 released in cubic millimeters per minute		
minutes			
2	0	11.4	102.7
3	0	10.0	71.4
4	0	8.2	61.2
10	0	9.4	10.8
11	0	8.8	9.2
12	0	10.2	6.8
13	0	8.2	
14	0	8.8	4.8
15	0	8.2	3.2
16	0	9.6	3.6
17	0	8.0	
18	0	7.8	1.0
19	0	7.6	1.8
20	0	8.2	0.6

exhibited a catalytic activity which was inhibited by cyanide, the activity must have been due to the enzyme. It is evident that the destruction of the mechanism of oxygen absorption is not related to the activity of catalase.

Experiment 2

Some investigators believe that the lysing principle is a living organism, and since all living matter contains catalase the sug-

gestion might well be made that the catalytic activity observed in the foregoing experiment was due to the bacteriophage itself and not to the presence of bacterial residue. The fact that it is possible to vary the amount of bacterial residue and yet maintain an equal final concentration of bacteriophage particles allows this point to be critically tested. It has been shown by many workers that at the time of complete lysis of cultures, the clear solutions always contain a concentration of bacteriophage particles of the same order of magnitude. In the present work this final concen-

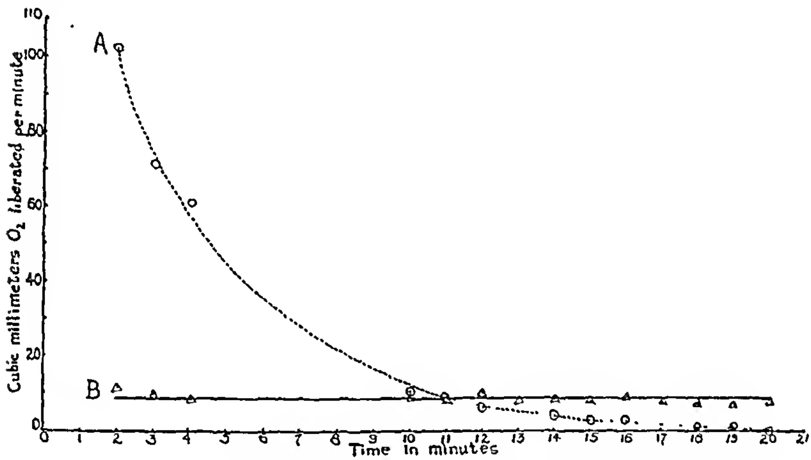

FIG. 1. THE RATE OF EVOLUTION OF O_2 BY CATALASE OF LYSED BACTERIAL CELLS

See table 1 for data. *A*, lysed cells; *B*, lysed cells plus KCN.

tration was determined by many titrations to be 10^9 active particles per milliliter of lysate.

In experiment 2, cultures were prepared as in experiment 1, except that one culture was lysed by the addition of 10 particles of bacteriophage per milliliter, another by 10^3 and the third by 10^5 particles per milliliter respectively. Under each condition, the multiplication of bacteria prior to the time of complete lysis will reach widely different levels and, in general, the number of bacteria will be higher in the cultures receiving lower concentrations of phage. The concentration of bacterial residues present after lysis will, therefore, be greater in the culture lysed by the smaller initial concentration of bacteriophage, although the final concen-

tration of bacteriophage will be equal in all solutions.[3] The data in table 2 show the arrangement of the experiment. From table 2 and figure 2, it is seen that the catalytic activity in the

TABLE 2

The rate of liberation of O_2 from H_2O_2 by the filtered lysates from cultures lysed with different initial concentrations of phage

	MANOMETER 1	MANOMETER 2	MANOMETER 3
Concentration in particles per ml. of phage used to induce lysis	10^5	10^3	10
0.75 per cent H_2O_2 at pH = 8.0	2.0 ml.	2.0 ml.	2.0 ml.
Filtrate of lysed culture	0.2 ml.	0.2 ml.	0.2 ml.
Buffer at pH = 8.0	10.0 ml.	10.2 ml.	10.0 ml.
Time	O_2 released in cubic millimeters		
minutes			
1	17.6	22.8	
2	13.2	18.8	66.8
3	8.2	13.4	59.2
4	5.4	10.8	48.6
5	4.0	7.6	45.4
6	3.0	7.2	33.2
7	3.2	5.2	29.0
8	2.4	4.8	21.4
9	1.8	3.6	18.2
10	0.8	3.4	22.8
11	1.6	2.4	10.6
12	0.6	2.6	11.4
13	0.6	2.0	8.8
14	1.0	1.0	
15	0.4	1.4	8.4
16	0.4	0.8	6.2
17	0.4	0.6	4.6
18		0.4	7.0
19		0.6	3.2

lysed culture varies according to the concentration of bacterial residue. Table 3 and figure 3 show that the rate of oxygen release decreases logarithmically, indicating the unimolecular nature of the reaction. As the reaction nears completion, the values

[3] At least when tested by the tenfold dilution method of Appelmans.

deviate somewhat as has been frequently observed in this type of reaction.

Experiment 3

The data of experiments 1 and 2 indicate the presence of the enzyme in a very labile condition, since its activity completely stops long before the total amount of available hydrogen peroxide

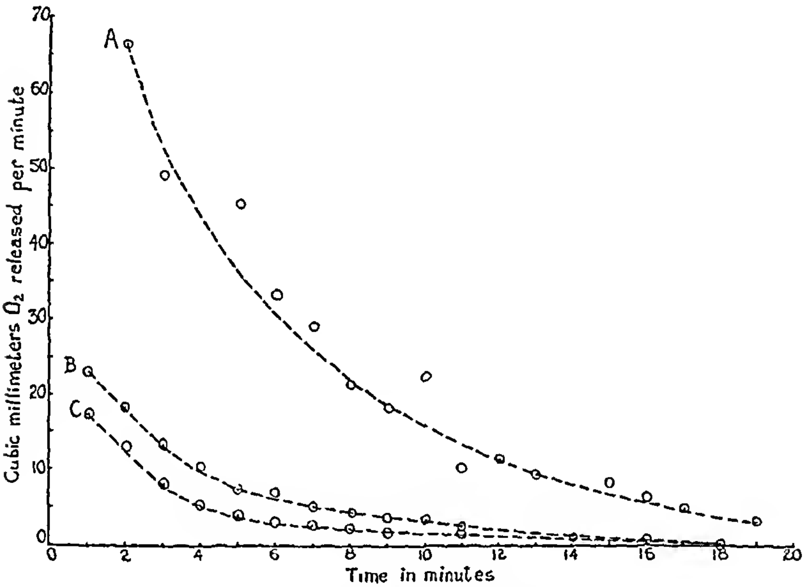

FIG. 2. CATALASE ACTIVITY OF LYSED CELLS

See table 2 for data. *A*, culture lysed with 10 particles of bacteriophase per ml. *B*, culture lysed with 10^3 particles per ml. *C*, culture lysed with 10^5 particles per ml.

has been destroyed. Experiment 3 was designed to test the effect of the concentration of the hydrogen peroxide on the degree of this lability. Cultures were prepared as described above and lysed with 10 particles of bacteriophage per milliliter. The clear solutions were then filtered through a Berkefeld filter and the catalase activity was measured in the presence of different amounts of hydrogen peroxide as shown in table 4. The rates of catalysis given in table 4 and figure 4 show that the rate of

oxygen evolution is initially greater with the higher concentrations of hydrogen peroxide, but that this rate declines and approaches zero much faster than when low concentrations of peroxide are used. Table 5 and figure 5 show that these rates are dependent on a unimolecular reaction.

TABLE 3

Logarithms of the catalase activities recorded in table 2 for experiment 2

These values are graphically presented in figure 3

Log_{10} of cubic millimeters of O_2 released per minute

TIME	MANOMETER 1	MANOMETER 2	MANOMETER 3
minutes			
1	1.25	1.36	
2	1.12	1.27	1.82
3	0.91	1.13	1.77
4	0.73	1.03	1.69
5	0.60	0.88	1.66
6	0.48	0.86	1.52
7	0.51	0.72	1.46
8	0.38	0.68	1.33
9	0.26	0.56	1.26
10		0.53	1.36
11	0.20	0.38	1.03
12		0.42	1.06
13		0.30	0.94
14	0.00	0.00	
15			0.92
16			0.79
17			0.66
18			
19			0.50

Experiment 4

From an inspection of figure 4, it appears possible that the total oxygen released is of the same order of magnitude irrespective of the initial concentrations of peroxide. In order to test this, cultures were prepared as for experiment 1, subjected to lysis by the addition of 10 particles of bacteriophage per milliliter and, subsequently, filtered through a Berkefeld filter. The ex-

periment was arranged as shown in table 6. Each solution contained 9 ml. of buffer at pH = 8.0, 0.2 ml. of lysed bacteria, and 2 ml. of hydrogen peroxide of various concentrations at pH = 8.0. The preparations were allowed to stand for one hour in the presence of the peroxide. After this time all catalytic activity was lost. The remaining hydrogen peroxide was determined by titra-

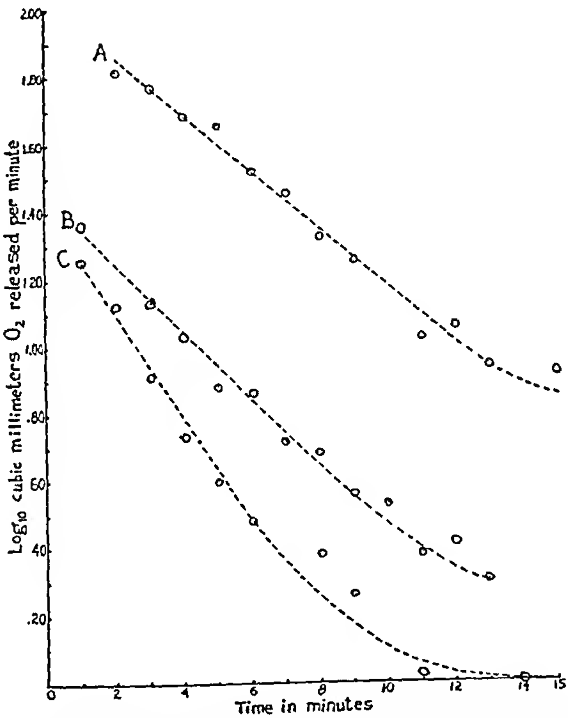

FIG. 3. UNIMOLECULAR NATURE OF THE RATE OF O_2 LIBERATION

See table 3 for data. *A*, culture lysed with 10 particles of bacteriophage per ml. *B*, culture lysed with 10^3 particles per ml. *C*, culture lysed with 10^5 particles per ml.

tion with 0.05 N potassium permanganate in the presence of sulphuric acid. The data recorded in table 6 show that aliquots of lysed cells destroy equal amounts of peroxide, regardless of the available concentration of peroxide. The only difference is that the release of the oxygen is accomplished in a shorter time at the higher peroxide concentrations.

TABLE 4

The rate of liberation of O_2 from H_2O_2 by the filtered lysate in the presence of different concentrations of H_2O_2

	MANOMETER 1	MANOMETER 2	MANOMETER 3
Concentration of H_2O_2	0.3 per cent	0.15 per cent	0.07 per cent
Volume of H_2O_2 added from the side arm	2.0 ml.	2.0 ml.	2.0 ml.
Filtrate of lysed culture......	0.2 ml.	0.2 ml.	0.2 ml.
Buffer at pH = 8.0..........	10.0 ml.	10.0 ml.	10.0 ml.
Time	O_2 released in cubic millimeters per minute		
minutes			
1		74.4	
2	106.4	70.0	36.0
3	101.0	54.0	30.0
4	54.6	46.0	29.0
5	41.4	48.0	26.0
7	27.4	29.6	23.4
8	13.4	24.4	19.2
9	13.4	19.6	14.6
10	9.4	15.8	16.6
11	7.2	13.2	13.4
12	6.2	10.8	12.4
13	4.0	9.6	9.8
14	3.0	8.2	9.4
15	2.6	6.2	7.8
16	2.4	4.8	7.6
17	2.0	3.6	7.6
18	0.6	2.8	4.8
19	1.2	3.4	6.4
20	1.2	2.8	4.8
21	0.2	2.6	4.4
22	0.6	2.8	4.0
23	0.4	0.6	
24	0.2	2.2	3.0
25	0.4	1.4	3.0
26	0.4	0.2	2.4
27	0.6	1.0	2.4
28	0.6	1.6	1.8
29	0.6	0.6	2.2
30	0.6	0.6	1.4

The results of experiment 4 show that the catalase present in the filtrate of the *Staphylococcus aureus* cells after lysis with bacteriophage is in an extremely labile condition. Even at the comparatively low concentration of peroxide used, the enzyme appears to bear a stoichiometrical relation to the destruction of hydrogen peroxide and, therefore, in the strictest sense, is not functioning as an enyzmatic catalyst at all but is engaging apparently in an irreversible reaction as one of the components.

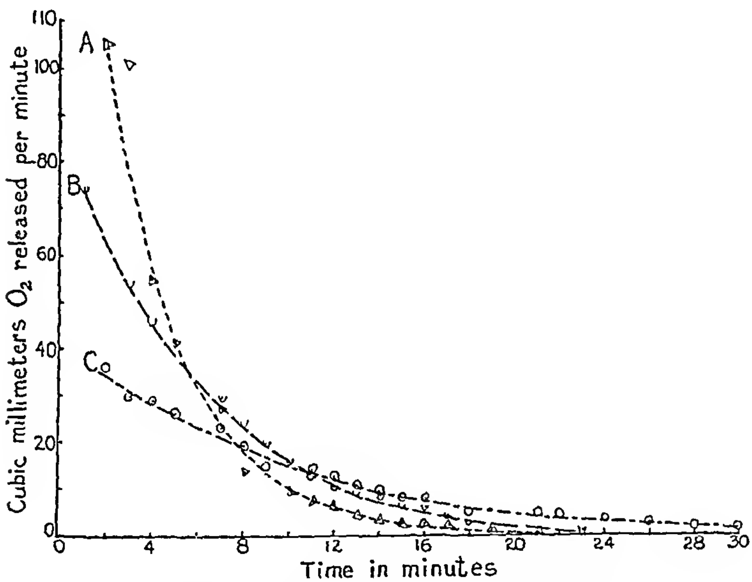

FIG. 4. CATALASE ACTIVITY OF LYSED CELLS

See table 4 for data. *A*, in presence of 0.3 per cent H_2O_2. *B*, in presence of 0.15 per cent H_2O_2. *C*, in presence of 0.07 per cent H_2O_2.

The rate at which H_2O_2 is destroyed (Figures 3, 5, 6) indicates this to be a unimolecular reaction. If the single component of the reaction capable of changing significantly be considered to be the substrate, (H_2O_2) then the activity constant is that of the catalytic reaction. However, it is evident from experiments 3 and 4 that the reaction stops completely when a certain amount of activity has taken place, irrespective of the amount of available substrate. The stoichiometrical nature of this reaction indicates that the amount of active enzyme is the component whose change

TABLE 5

Logarithms of catalase activities recorded in table 4 for experiment 3
These values are graphically presented in figure 5

TIME	LOG10 OF CUBIC MILLIMETERS OF O_2 RELEASED PER MINUTE		
	Manometer 1 0.3 per cent H_2O_2	Manometer 2 0.15 per cent H_2O_2	Manometer 3 0.07 per cent H_2O_2
minutes			
1		0.87	
2	2.03	1.85	1.56
3	2.00	1.73	1.48
4	1.74	1.66	1.46
5	1.62	1.68	1.42
7	1.44	1.47	1.37
8	1.13	1.39	1.28
9	1.13	1.29	1.28
10	0.97	1.20	1.22
11	0.86	1.12	1.13
12	0.79	1.03	1.09
13	0.60	0.98	0.99
14	0.48	0.91	0.97
15	0.42	0.79	0.89
16	0.38	0.68	0.88
17	0.30	0.56	0.88
18		0.45	0.68
19	0.08	0.53	0.81
20	0.08	0.45	0.68
21		0.42	0.64
22		0.45	0.60
24		0.34	0.48
25		0.15	0.48
26			0.38
28			0.26
30			0.15

in concentration imparts the unimolecular nature to the reaction. The constant, k, in the familiar equation for the reaction rate of a unimolecular reaction

$$k = \frac{1}{t} \log_e \frac{a}{a - x}$$

is, therefore, the reaction constant for the rate of the *inactivation* of the enzyme and not directly that of the enzymatic catalytic activity. Also, the symbol *a* refers to the concentrations of the enzyme and not of the substrate.

Several authors have described the failure of catalase to produce a typical monomolecular catalytic reaction. Senter (1903) reported that monomolecular catalysis is possible only at very low concentrations of hydrogen peroxide and at low temperatures.

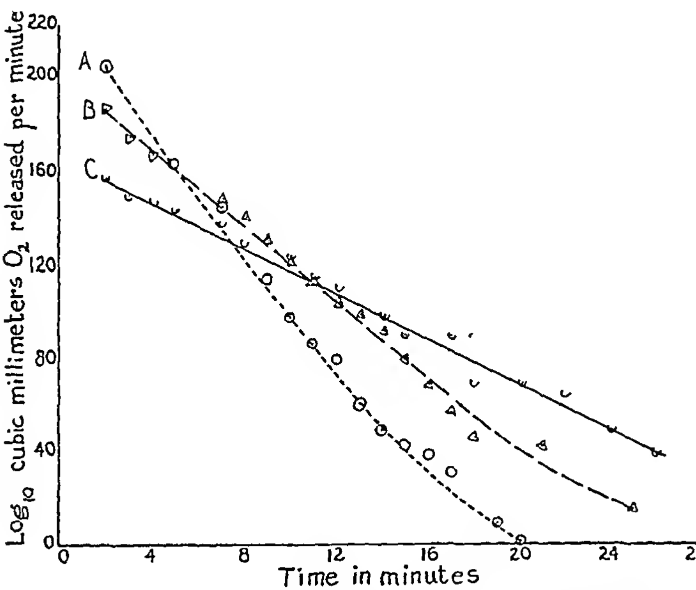

FIG. 5. UNIMOLECULAR NATURE OF THE RATE OF O_2 LIBERATION
See table 5 for data. *A*, in the presence of 0.3 per cent H_2O_2. *B*, in the presence of 0.15 per cent H_2O_2. *C*, in the presence of 0.07 per cent H_2O_2.

Morgulis (1921) found that the enzyme was rapidly destroyed in high concentrations of peroxide at 20°C. Northrop (1925), working with catalase preparations from liver, found that the results of Margulis could be explained by the fact that the rate of decomposition of peroxide is proportional at any time to the concentration of the enzyme, and that catalase decomposes by a monomolecular reaction which is independent of the concentration of catalase, and is inversely proportional to the original concen-

TABLE 6

The amount of O_2 released from H_2O_2 by the filtered lysate in the presence of different concentrations of H_2O_2

	SERIES 1	SERIES 2	SERIES 3	SERIES 4
Buffer at pH = 8.0	9 0 ml.	9 0 ml.	9 0 ml.	9 0 ml.
Filtrate of lysed culture	0 2 ml.	0 2 ml.	0 2 ml.	0.2 ml
Initial concentration of H_2O_2	0 6%	0 3%	0 15%	0.07%
Per cent of the initial H_2O_2 destroyed	6 3%	10.8%	27 8%	48.2%
H_2O_2 destroyed in equivalents....... . .	22.2 x 10^{-5}	24.2 x 10^{-5}	24.7 x 10^{-5}	21 7 x 10^{-5}

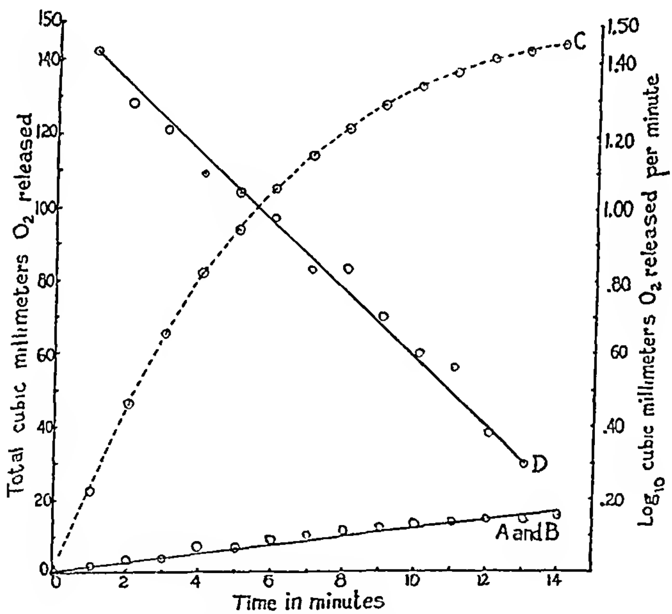

FIG. 6. THE EFFECT OF DIFFERENT CONCENTRATIONS OF KCN ON CATALASE ACTIVITY OF LYSED CELLS

See table 7 for data The right hand ordinates refer to curve *D*. *A*, in the presence of 0 1 per cent KCN. *B*, in presence of 0 2 per cent KCN. Curves *A* and *B* are completely superimposed. *C*, O_2 evolution in absence of KCN. *D*, Log_{10} of rate of O_2 evolution showing unimolecular nature of the reaction.

tration of hydrogen peroxide up to 0.4 M. He expressed the reaction constant as,

$$K = \frac{1}{t} \log \frac{A}{A - x},$$

where x is the oxygen liberated in time t, A is the total amount of O_2 liberated when the reaction has ceased and not the total amount of oxygen available in the peroxide, and K is the inactivation constant of the enzyme.

TABLE 7

The amount of O_2 released from H_2O_2 by the filtered lysate in the presence of different concentrations of KCN

	MANOMETER 1	MANOMETER 2	MANOMETER 3	RATE OF THE RELEASE OF O_2 PER MINUTE OF THE CONTROL CULTURE IN MANOMETER 3	LOG_{10} OF RATE OF RELEASE OF O_2 BY THE CONTROL CULTURE IN MANOMETER 3
Buffer at pH = 8.1	9 ml.	9 ml.	9 ml.		
Filtrate lysate	0.2 ml.	0.2 ml.	0.2 ml.		
Broth.			1.0 ml.		
1 per cent KCN at pH = 8.1.		1.0 ml.			
2 per cent KCN at pH = 8.1.	1.0 ml.				
Final Concentration of KCN........	0.2 per cent	0.1 per cent			
Time	O_2 released in cubic millimeters				
minutes					
1	2.0	2.0	22.0		
2	3.6	3.4	46.2	26.2	1.42
3	4.0	5.0	65.4	19.2	1.28
4	7.0	6.2	81.8	16.4	1.21
5	6.8	7.2	94.0	12.2	1.10
6	9.0	8.2	105.0	11.0	1.04
7	10.2	9.8	114.4	9.4	0.97
8	11.2	10.6	121.2	6.8	0.83
9	12.0	11.2	125.0	6.8	0.83
10	13.2	13.2	133.0	5.0	0.70
11	13.8	14.2	137.0	4.0	0.60
12	14.6	14.4	140.0	3.6	0.56
13	15.0	15.8	143.0	2.4	0.38
14	15.8	16.8	145.0	2.0	0.30

This interpretation is essentially that described by the present authors, and it is significant in showing that preparations of catalase, more or less free from intimate combination with cellular structures, are in a very labile condition and do not give a typical reversible enzymatic catalysis.

Experiment 5

Figure 1 indicates that lysed cells are able to free oxygen from the hydrogen peroxide at a low but constant rate in the presence of cyanide. This is not due to any reaction of the medium since control broth showed no perceptible catalytic activity (table 1) and it must be due to some cyanide-stable component of the lysed culture. In order to show that this activity was not due to a partial recovery from the effects of the cyanide, such as may result from the cyanide distilling into the alkali contained in the inset of the reaction vessel, experiment 5 was arranged as indicated in table 7. The pH was buffered near 8.0 as in the previous experiments to lessen the tendency of the hydro-cyanic gas to escape. The data presented in table 7 and figure 6 show that the two concentrations of cyanide gave identical results. If there had been a recovery due to cyanide distillation, the solution containing the most cyanide would have lagged behind the others.

SUMMARY

1. Cultures of *Staphylococcus aureus* lysed by lacteriophage exhibit no utilization of oxygen.

2. Lysis by bacteriophage does not destroy the catalase activity of this organism.

3. The catalase present in the filtrate of lysed cells is in a very labile condition, and it appears to destroy hydrogen peroxide by an irreversible stoichimetrical reaction.

4. This catalase reaction is inhibited by cyanide.

5. The destruction of hydrogen peroxide proceeds in accordance with the equation for a unimolecular reaction and the reaction constant is that of the *rate of inactivation of the enzyme*, and not that of the disappearance of the substrate.

6. The similarity of the present results with those obtained by Northrop on liver preparations shows that when catalase is more or less freed from intimate association with cellular structures it is unable to enter reversibly into the catalyzed reaction.

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A MODIFIED FERMENTATION TUBE

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The drawing reproduced below depicts the essential features of a type of fermentation tube designed to replace for some purposes the usual form in which an inverted vial collects the gas evolved. In so far as the writer is aware, tubes designed after the principle involved in this one have not hitherto been described; certainly they are not in common use, simple though they be.

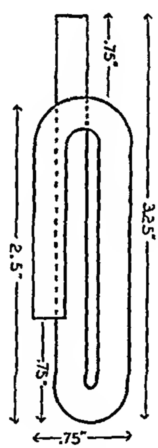

Fig. 1

The tube shown is made of Pyrex glass tubing of 6 mm. outside diameter, 1 mm. wall thickness, and the dimensions are such as to fit a standard 100 ml. water sample bottle or a 25 mm. culture tube. Of course, within limits, any size of glass tubing can be used and the dimensions varied to suit the individual case.

The essential features are obvious. Both ends are open, the

upper end must extend some distance above the upper bend, and it is best that the lower end terminate above the lower bend.

The great advantage of this tube is that it fills when dropped into liquid, or, if in an empty vessel, fills when fluid is poured in. When once filled, both arms of the inverted "U" serve as traps for any gas evolved.

Tubes of this sort would seem to offer advantages for certain phases of public health bacteriology. Although both dehydrated and very concentrated media have been found to change in sterilization, lactose broth of seven times normal strength can be placed in a sample bottle together with a tube. With such a culture system the volume of the bottle need be little larger than that of the water sample, and there is no danger of admitting air to the gas tube either through tipping or drying. It would seem feasible, particularly where some time must elapse before samples can reach the laboratory, to utilize this time for growth and gas production.

It is not infrequently desired to run a fermentation or two with some carbohydrate which is not at the moment ready for use. If some tubes of the kind described are sterilized separately and kept on hand, it is a matter of minutes only to prepare the carbohydrate solution, filter to sterilize, add the desired amount to the nutrient base, inoculate, and drop in the sterile fermentation tube: no time need be wasted in autoclaving. The same procedure could be used when autoclaving was contraindicated because of possible damage to the carbohydrate.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK STATE BRANCH

38th Semi-Annual Meeting, Geneva, New York, October 28, 1939

Precipitates in the New Standard Agar. *G. J. Hucker*, Division of Bacteriology, New York State Experiment Station, Geneva, New York.

The Use of Quinhydrone Electrode for Correlating Final H-Ion Concentration and Pigmentation of Actinomycetes Growing on Agar. *Jean E. Conn*, Division of Bacteriology, New York State Agricultural Experiment Station, Geneva, New York.

In a study of the pigmentation of Actinomycetes, the need arose for a method of determining the final H-ion concentration of cultures growing on agar. The quinhydrone electrode was found to be suitable for this purpose when the agar was handled as follows. The slanted column of agar was forced out of the test-tube by heating the base. Then, after macerating with quinhydrone, the mixture was placed in a section of a drinking straw and affixed to the electrode by means of a paper-fastener. The final H-ion concentration could then be determined in the usual way.

This method was used for four species of pigmented *Actinomyces* which were grown on a glycerol-asparagin medium to which amounts of glucose varying from 0.1 to 5.0 per cent had been added. They were selected because they showed marked changes in the color of their pigments. The results indicated complete agreement between these color changes and changes in final H-ion concentration. It would appear, therefore, that the pigments of these cultures act as indicators, changing respectively from blue to red, violet to red, bright red to pink, and black to light brown, as the acidity of the medium increases.

The Effect of Sulfanilamide and of Sulfapyridine on Toxic Substances from Lysed Pneumococci, Type I. *C. M. Carpenter, G. M. Barbour and Helen Ackerman*, University of Rochester, Rochester, New York.

Microbiological Flora of Pulp, Paper and Paperboard. *J. R. Sanborn and Raphael A. Gillotte*, Division of Bacteriology, New York State Agricultural Experiment Station, Geneva, New York.

The micro-organisms, which are present in pulp, paper and paperboard, have their origin in the water supplies, and the pulpwood and other raw materials that are employed in the process of manufacturing. Organisms, also, may be transferred directly from the growths of capsulated, gum-producing forms which previously have become established in mill pipe-lines and on

equipment. The various groups of the predominating species encountered have been isolated, cultivated and identified. These belonged to the genera, *Bacillus*, *Aerobacter*, *Proteus*, *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Sarcina*, *Actinomyces*. The fungi which occurred most frequently were members of the genera, *Aspergillus*, *Penicillium*, *Trichoderma*, *Alternaria*, *Oidium*, *Botrytis*, *Cladosporium*, *Fusarium*, *Chaetomium*.

Pulp, paper and paperboard are subjected during manufacture to physical and chemical treatments which usually eliminate all but the more resistant forms.

About 90 per cent of the organisms isolated from finished paperboard are aerobic, spore-forming bacilli and micrococci. The remaining types are sarcinae, actinomyces, non-spore forming rods and filamentous fungi. A variety of fungi develop in pulp mills, but these types occur infrequently in finished paper and paperboard. A few persistent mold-spores may survive.

MORPHOLOGY AND GROWTH OF THERMOPHILES DURING FERMENTATION OF CELLULOSE. *T. S. Polansky and R. W. Stone*, Division of Bacteriology, Pennsylvania State College, State College, Pennsylvania.

Three types of thermophilic organisms were isolated from horse manure by means of enrichment cultures in deep tubes incubated at 60°C. The first type produced an odor of hydrogen sulfide, the second a yellow pigment, and the third no pigment. The organisms were differentiated by their action on filter paper and by the products formed.

Morphology was studied by making gram stains and Fulton-Schaeffer spore stains daily. Young cultures were gram-positive. Older cultures gradually became gram-negative with the appearance of terminal spores that resembled large cocci and coccoid forms. At certain stages of growth the stains showed what appeared to be mixed cultures of large gram-positive rods, smaller gram-negative rods and coccoid forms. However, heating the cultures at 100°C. for one-half hour did not alter their morphological characteristics upon subsequent cultivation. All were motile and had peritrichous flagella.

An organic source of nitrogen such as peptone was found necessary for the decomposition of cellulose. Small amounts of Fe^{++} and Sn^{++} had a stimulatory effect.

RESISTANCE TO BACTERIAL WILT OF MAIZE AND THE GENETIC HOST-PARASITE RELATIONSHIP. *R. Lincoln*, Department of Plant Pathology, College of Agriculture, Cornell University, Ithaca, New York.

BLACK-ROT OF BARBAREA VULGARIS AND ITS RELATION TO THE BLACK-ROT OF CABBAGE. *W. H. Burkholder*, Department of Plant Pathology, College of Agriculture, Cornell University, Ithaca, New York.

THE ISOLATION OF ACTINOMYCES MURIS FROM A CASE OF RAT-BITE FEVER. *Alice D. Leahy and Fred S. Gachet*, Departments of Bacteriology and Pediatrics, University of Rochester School of Medicine and Dentistry, and Strong Memorial Hospital, Rochester, New York.

A 14-year-old boy was admitted on September 2, 1939 to the Rochester Municipal Hospital with a history of a rat bite six days previously. Twenty-four hours before admission, headache, malaise, nausea, and vomiting had developed. Physical examination on admission revealed a tempera-

ture of 39.7°C. and numerous discrete maculo-papular spots, about 2 mm. in diameter, scattered over the extremities. The exanthem, where it occurred, resembled that of rubella. In the absence of a local lesion, a tentative diagnosis of Haverhill fever was made.

A filamentous, gram-negative organism, which grew in broth cultures but not in poured plates, was isolated from six blood cultures taken at three-day intervals. Upon subculture, it grew abundantly in ascitic fluid broth, but not in plain Douglas's broth. Growth on rabbit's blood, "chocolate", Levinthal's and Bradford's plates was slight. The organism was non-motile and non-acid-fast.

Intra-abdominal inoculation of white mice gave the results recorded below. Five-tenths ml. of the patient's blood produced no disease in nine days, and the organism was not recovered at autopsy. One ml. of an ascitic-fluid broth culture produced arthritis on the seventh day and death on the thirteenth. The organism was recovered from the synovial fluid and heart's blood, and injected into a series of mice. All died in from 24 to 48 hours. Cultures from the peritoneal cavity, liver, spleen, and heart's blood were positive. Morphologically and culturally, the organism appeared similar to, if not identical with, *Actinomyces muris*. Its isolation from the patient's blood in repeated cultures, therefore, confirmed the clinical diagnosis.

REVIEW OF THE WORK OF THE INTERNATIONAL COMMITTEE ON NOMENCLATURE, THIRD INTERNATIONAL CONGRESS FOR MICROBIOLOGY. *Robert S. Breed*, Division of Bacteriology, New York State Agricultural Experiment Station, Geneva, New York.

THE ANTIGENIC RELATIONSHIP OF SHIGELLA ALKALESCENS TO OTHER AEROBIC, GRAM-NEGATIVE BACTERIA. *Erwin Neter*, Children's Hospital and University of Buffalo, Buffalo, New York.

After 33 strains of *Shigella alkalescens* (Andrewes) had been identified as Type I of de Assis by their uniform biochemical reactions and antigenic homogeneity, the antigenic relationship of *Shigella alkalescens* to other aerobic, gram-negative bacteria was studied. The macroscopic agglutination and agglutinin-absorption techniques were employed with an anti-*Shigella-alkalescens* rabbit serum of high titer (1:10,000) as the immune serum and the respective organisms in formalinized suspensions as the antigens. The agglutination tests were read after 18 hours incubation at 55°C.

The results follow: 1) *No agglutination: Aerobacter aerogenes*, 10 strains; *Proteus vulgaris*, 2 strains; *Proteus morgani*, 5 strains of Type I and 4 strains of Type XIV; *Pseudomonas aeruginosa*, 2 strains; and *Eberthella oedematiens* Assis, 1 strain. 2) *Positive agglutination* in high titer (1:10,000): 2 of 5 strains of *Eberthella belfastiensis* (*Escherichia anaerogenes*, as described by Bamforth) and 1 of 9 strains of *Escherichia coli* (atypical strain resembling *Escherichia coli-mutabile*).

When agglutinin-absorption tests were carried out, the two strains of *Eberthella belfastiensis* resulted in but slight reduction in the titer of agglutinins for *Shigella alkalescens* whereas the atypical *Escherichia coli* antigen almost completely removed the anti-*Shigella-alkalescens* agglutinins.

THE INFLUENCE OF BACTERIAL AND NON-BACTERIAL POLYSACCHARIDES UPON BACTERIOPHAGY. *N. J. Ash-*

enburg, L. A. Sandholzer and H. W. Scherp, Department of Bacteriology, University of Rochester, Rochester, New York.

THE USE OF SPORES AS ANTIGENIC MATERIAL IN THE SEROLOGICAL DIFFERENTIATION OF SPECIES WITHIN THE GENUS BACILLUS. *Carl Lamanna*, Department of Dairy Industry, College of Agriculture, Cornell University, Ithaca, New York.

An attempt has been made to develop a serological method for differentiating groups or species within the genus *Bacillus*.

The antigenic material was limited to spores only, in the hope that they would exhibit high concentrations of group or species-specific antigen. The results which indicate that spores are antigenically distinct from vegetative cells, are in accord with past findings. Furthermore, spores of different species differ antigenically. In some instances this finding has taxonomic value, as evidenced by the high correlation of morphological and physiological data with the serological data.

An agglutination test which involved shaking and the use of a concentrated spore-suspension proved most practicable. Spores were injected intravenously into rabbits to obtain antibody.

Three serological groups were found in the small-celled species:

1) *Bacillus subtilis* Group. Species: *Bacillus subtilis*—equatorial germination; nitrates reduced; glucose, sucrose, starch attacked; growth at 55°C., no growth at 15° and 10°C.

2) *Bacillus mesentericus* Group. Species: a) *Bacillus vulgatus*—equatorial germination with splitting along transverse axis; nitrates reduced; glucose, sucrose, starch attacked; growth at 50° and 15°C., no growth at 55° and 10°C. b) *Bacillus mesentericus*—germination by comma-shaped expansion; nitrates not reduced; glucose, sucrose fermented; starch not attacked; growth at 50° and 15°C., no growth at 55° and 10°C.

3) *Bacillus agri* Group. Species: *Bacillus agri*—polar germination; nitrates not reduced; glucose, sucrose, starch not attacked; growth at 55°C., no growth at 10° and 15°C.

THE EFFECT OF TEMPERATURE ON RESULTS SECURED WITH SOME MODIFICATIONS OF THE VOGES-PROSKAUER TEST. *S. Snieszko and J. Skorka*, Jagellonian University, Krakow, Poland.

Forty-six strains of *Escherichia coli* and *Aerobacter aerogenes* were isolated from milk and water. For enrichment purposes, milk was inoculated into broth containing 0.5 per cent glucose, 0.5 per cent K_2HPO_4, 0.5 peptone (Clark broth). Cultures were then isolated at 37°C. Those from water were isolated both at 28°C. and at 37°C. When isolated and identified, each strain was inoculated into ten test-tubes, containing Clark-broth; five of which were incubated at 28–29°C. and the other five at 36–37°C.

Beginning 24 hours after inoculation, one test tube of each strain at each of these incubation temperatures was tested with Baritt's and O'Meara's modifications of the Voges-Proskauer test. Methyl red and Koser's sodium citrate agar tests were also made.

It was found that *Aerobacter aerogenes* could be more easily isolated from water at 28°C. than at 37°C. Complete agreement with results secured by Vaughn, Mitchell and Levine* was noted. An incubation temperature of 28–29°C. was much more favor-

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able than 37°C. for detecting differences between *Escherichia coli* and *Aerobacter aerogenes*. The Barrit test proved better and quicker than any other test used.

BACTERIAL GROWTH IN MILK AS A POSSIBLE SOURCE OF ERROR IN THE PHOSPHATASE TEST. *Harold W. Leahy, Leslie A. Sandholzer, and Marian R. Woodside*, Rochester Health Bureau Laboratories, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

Twenty-eight strains of bacteria, belonging to the following genera, *Lactobacillus, Streptococcus, Staphylococcus, Escherichia, Aerobacter, Salmonella, Klebsiella,* and *Pseudomonas,* have been tested for their ability to produce phosphatase when grown in milk. One strain of *Staphylococcus*, five strains of *Aerobacter*, and three strains of *Klebsiella* produced a phosphatase which showed slight activity at pH 9.0. Seven additional strains of various genera (*Lactobacillus, Streptococcus, Escherichia, Salmonella, Aerobacter,* and *Pseudomonas*) gave a false phosphatase reaction, when Folin-Ciocalteu's reagent was employed.

A comparison of Folin-Ciocalteu's reagent with the phenol reagent of Gibbs demonstrated the superiority of the latter. Folin-Ciocalteu's reagent gave rise to as strong colors with ketones, hydroxy-ketones, indol derivatives, oxygenated purine derivatives, amino acids, and several inorganic ions as with phenol. It is suggested that the false phosphatase reactions obtained with the Kay-Graham method are due to the hydrolysis of the bacterial nucleic acids to oxypurines. Phosphatase tests made upon milks having microscopic counts higher than 8,000,000 per ml., or standard plate counts higher than 2,000,000 per ml., should be interpreted with caution.

INDEX TO VOLUME 38